DISCOVERY OF NOVEL MOLECULAR IMMUNE MEDIATORS IN THE AMERICAN LOBSTER (*Homarus americanus*) DURING BACTERIAL, EUKARYOTIC PARASITIC AND VIRAL CHALLENGES

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Abstract

The American lobster (*Homarus americanus*) is the most economically significant commercial fishery species in Canada. The Canadian lobster fishery provides tens of thousands of jobs, and is the economic driver of hundreds of rural communities in Atlantic Canada and Quebec. The adult lobster is susceptible to relatively few pathogens but very little is known about how its immune system is capable of mediating this pathogen resistance. Additionally, there is no definitive clinical biomarker that is capable of assessing overall lobster health, something that would be valuable to a fishery where lobsters are the most lucrative when they are sold as a live product. The purpose of this project was to discover how the *H. americanus* humoral immune system responds to bacterial, eukaryotic parasitic and viral pathogens. This was performed by using high-throughput transcriptomics, in the form of microarray gene-expression analysis, to monitor the expression of classical immune molecules, as well as discover novel immune mediators. This project discovered hundreds of new *H. americanus* genes that have not previously been associated with either lobster or crustacean immunity. The expression of these novel immune-related genes is especially interesting because over half of them have no similarity to proteins in GenBank, little if any functional characterization, and pathogen class-specific expression. The conventional immune paradigm for any crustacean innate immune response is that it is non-specific and responds similarly to all microorganisms, pathogenic or not. These studies have determined that this is not the case. There is consistent and unique differential expression of genes for each of the pathogen classes examined, and even differential expression of isoforms within gene families that is pathogen dependent. Among the most interesting genes that have been discovered are the six isoforms of anti-lipopolysaccharide factor family (ALFHa-1, ALFHa-2, ALFHa-3, ALFHa-4, ALFHa-6 and ALFHa-7), acute phase serum amyloid protein A (SAA) and trypsin 1b. Four ALFHa family isoforms are differentially expressed during bacterial, *Aerococcus viridans* var. *homari*, and parasitic, *Anophryoides haemophila*, infections, where the differential expression of ALFHa-4 and ALFHa-2 isoforms are the most significant during bacterial and parasitic infections respectively. However, none of the six ALFHa genes are differentially expressed during viral, White Spot Syndrome Virus, infection. SAA is important because its expression is significantly increased in moribund lobsters during bacterial and parasitic infections, although not in viral infections. The protein sequence of SAA is highly conserved between humans and *H. americanus* suggesting the conservation of an important biological function such as innate immune activation. Trypsin 1b is the only gene that was differentially expressed during all three pathogen challenges. It is unclear what immunological role this gene plays in lobster or crustacean immunity but it is likely pivotal to immune activation, and a promising lobster health biomarker. These findings have made significant advances to our understanding of lobster immunity which can be applied to crustacean immunology as a whole. The lobster immune response can no longer be thought of a generalized non-specific response, but as one tailored to the invading pathogen.

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Dedication

TO SARAH AND RORY

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Chapter 1: General Introduction

1.1 Canadian Lobster Industry

The Canadian lobster fishery is the most economically important commercial fishery in Atlantic Canada, worth more than $1 billion annually. Hundreds of Atlantic Canadian coastal communities have relied on this fishery for over 100 years, where close to 10,000 individual lobster license holders are actively involved in the modern fishery. The targeted species of the Canadian lobster fishery is the American lobster (*Homarus americanus*), and over 50,000 metric tonnes of live-lobster is landed each year. This represents one third of the total landed value of all commercial Canadian wild-fisheries (http://www.frcc.ca/2007/Strategic Lobster Framework 2007.pdf).

The Canadian lobster fishery is based primarily in Nova Scotia, New Brunswick and Prince Edward Island, but smaller fisheries exist in Newfoundland and Quebec. The natural geographic range of *H. americanus* is from North Carolina to the Labrador Sea and from the coastal waterline to the edge of the continental shelf. However, the highly productive lobster fishing grounds are in the Gulf of Maine, Southwestern New Brunswick, Southern Nova Scotia and the Southern Gulf of St. Lawrence (Pezzack, 1992). Most of the fishery is in shallow water less than 40 m deep (Duggan, 1985) where lobsters prefer rocky benthic habitat, over muddy or sandy substrates, as it provides more opportunities for secure shelters.

The Canadian lobster fishery is managed within 45 lobster fishing areas (LFA) where each LFA has defined fishing seasons, minimal carapace length and trap limits to ensure the sustainability of the fishery. The harvesting regulations imposed within each LFA have been established based on biological, socioeconomic, political and climatic characteristics unique to each particular LFA.

1.2. American Lobster Biology

Lobsters live at least 5-8 years in the wild before they are eligible for recruitment into the fishery. Different size restrictions and water temperatures throughout Atlantic Canada result in geographic differences in age at recruitment where 5-6 years is typical in the Southern Gulf of St. Lawrence, and 6-8 years is typical on the Atlantic coast of Nova Scotia (Aiken, 1980). Early life stages of *H. americanus* consist of three larval and one post-larval pelagic stage. The post-larval stage engages in bottom-seeking behaviour where it searches for suitable benthic habitat and settles when it discovers appropriate habitat. If suitable benthic habitat cannot be found, the post-larva return to the water column to drift some distance away, and repeat the search for appropriate benthic substrate (Cobb et al., 1989). The pelagic life stages of *H. americanus* usually only last between two weeks and two months, with the remainder of their life spent in the benthic environment (MacKenzie, 1988; Ennis, 1995).

A juvenile life stage follows the larval lobster life stages. The juvenile life stages can be subdivided into an early shelter-dependent period, followed by progressively frequent movements outside of its protective shelter to explore the surrounding habitat (Wahle,

1992). Larger juveniles and adults are capable of travelling greater distances from their shelters in search of food due to the reduced risk of predation that comes with increased size.

1.3 Lobster Disease

The natural marine environment exposes lobsters to innumerable potential pathogens; however the adult lobster is relatively resistant to disease. The commercial lobster fishery targets the adult lobster population and consequently, most of the disease susceptibility information is restricted to wild adult, post-harvest adults, hatchery reared larval and early juvenile lobster. The significant diseases for the adult lobster are gaffkemia, caused by the Gram-positive bacteria *Aerococcus viridans* var. *homari* (Hitchner and Snieszko, 1947; Snieszko and Taylor, 1947; Stewart and MacDonald, 1962; Stewart, 1975; 1980), bumper-car disease, caused by the scuticociliate *Anophryoides haemophila* (Sherburne and Bean, 1991; Cawthorn et al., 1996; Cawthorn,1997) and chitinolytic shell-disease (Smolowitz et al., 2005). Unlike other decapod crustaceans, a naturally occurring viral pathogen of *H. americanus* has never been found.

1.3.1 Gaffkemia

Gaffkemia is the major warm water (>10 ^{o}C) disease of lobster. *Aerococcus viridans* is a free-living cocci bacterium that lacks exochitinase enzymes and is only able to infect lobsters by entering through a defect in the integument. Only 10 colony forming units per kg of lobster body weight are required to cause lethal disease, although the progression of gaffkemia is temperature dependent. Mortality from *A. viridans* infection can take up to

180 days at 3 ^{o}C and as few as 2 days at 20 ^{o}C (Stewart, 1984). The bacterium is cleared from the circulating haemolymph upon initial infection through phagocytosis by fixed phagocytes in the hepatopancreas, and circulating haemocytes (Stewart and Arie, 1981). *A. viridans* is able to overcome the cellular and humoral immune system of *H. americanus*, and rapidly proliferates in phagocytes until they have reached such numbers as to lyse the cells and cause a lethal systemic infection (Rabin 1965; Rabin and Hughes, 1968; Stewart et al., 1968; 1969a; 1969b; Stewart and Zwicker, 1972; Cornick and Stewart, 1968). Proliferation of *A. viridans* in lobster haemolymph is correlated with haemocytopenia (Stewart et al., 1969a).

There are no clinical signs of gaffkemia indicative of a definitive *A. viridans* infection diagnosis. Common signs are weakness and lethargy, poor defensive posture, anorexia and occasionally a red or pink discolouration of the haemolymph known as "red tail" (Stewart and Arie, 1973; Snieszko and Taylor, 1947). Moribund infected lobsters also have severely impaired clotting ability likely due to haemocytopenia (Stewart et al., 1969a; Johnson et al., 1981). Heavy infections of *A. viridans* in moribund lobsters can be detected by Gram stains of haemolymph smears which reveal Gram-positive tetrads (Stewart, 1980).

Aerococcus. viridans is endemic to the common lobster fishing grounds, and has been found at rates of 1-32% in the wild (Rabin, 1965; Stewart et al., 1966; Vachon et al., 1981; Lavallée et al., 2001). However, this number is widely believed to be an underestimate of the true infection rate as these prevalence studies are based on trap

surveys and infected lobsters quickly lose the desire to forage for food (Lavallée et al., 2001). A survey of wild-caught European lobster, *Homarus gammarus*, off the coast of Britain has also found evidence of *Aeroccus viridans* infections in the wild (Stebbing et al., 2012). *A. viridans* can survive for extended periods of time in the marine benthos and is an especially persistent cause of mortality to impounded lobsters because of its ability to form biofilms on live-lobster holding infrastructure (Cawthorn, 2011).

1.3.2 Bumper Car Disease

Bumper-car disease is caused by the protozoan scuticociliate parasite *A. haemophila* (Cawthorn et al., 1996; Cawthorn, 1997; Lavallée et al., 2001; Greenwood et al., 2005). *A. haemophila* prefers cold temperatures (< 5 ^{o}C) (Aiken et al., 1973; Cawthorn, 1997; Loughlin et al., 1993) but has also been found in lobsters maintained for three to four weeks at temperatures as high as (10 ^{o}C) (personal observation). Infection in wild and impounded lobsters was initially reported in Maine in 1990 (Sherburne and Bean, 1991); however, it wasn't until 1996 that the ciliate was identified as *A. haemophila* (Cawthorn et al., 1996). Since this time, *A. haemophila* has been found in impounded lobsters in Nova Scotia, and wild lobsters around Prince Edward Island (Lavallée et al., 2001; Greenwood et al., 2005).

The route of *A. haemophila* infection is believed to be through defects in the lobster carapace (Sherburne and Bean, 1991), through the thin cuticular covering of the gills or through the new shell of a recently moulted lobster (Cawthorn, 1997). An alternative hypothesis is that *A. haemophila* is capable of active penetration of the lobster

exoskeleton using the secretion of proteases. *Anophryoides haemophila* contains several cysteine proteases including several isoforms of cathepsin B and L. However, *in vitro* experimentation of *A. haemophila* in the presence and absence of different concentrations of lobster haemolymph did not find differential expression of cathepsin B1, B2 and L1 isoforms (Acorn et al., 2011). This suggests that *A. haemophila* does not increase the expression of these cysteine proteases as a means of host invasion via this mechanism. This however, has not ruled out other potential secreted proteases for host invasion.

Bumper-car disease progression occurs in two stages. The initial stage lasts 1 - 4 weeks, lacks clinical signs, and *A. haemophila* is absent from circulating haemolymph. During this time, haemocyte aggregations can be found in the gills and vesicular spaces with a ciliate occasionally found in the centre of these aggregations. After the fourth week of infection, systemic progression of the disease is concomitant with ciliate proliferation and haemocytopenia. *A. haemophila* infections are usually lethal by week 14 (Sherburne and Bean, 1991; Athanassopoulou et al., 2004).

1.3.3 Shell Disease

There are at least three different forms of shell disease that manifest in *H. americanus*. The first is impoundment shell disease, initially recognized in the 1930s (Hess, 1937). Impoundment shell disease is associated with maintaining lobsters in poor conditions including poor water quality, overcrowding and inadequate diet (Prince et al., 1995). Round black lesions form on the lobster carapace and progressively overlap and amalgamate as the disease progresses (Bullis, 1989; Fisher, 1988).

The second type of shell disease is burnt (rust) spot shell disease. This form of shell disease is found on lobsters harvested from offshore canyons and is normally associated with pollution (Sindermann, 1989; Ziskowshi et al., 1996). Multifocal circular black lesions begin at carapace pores, or pits, and are dispersed across the lobster carapace (Stewart, 1980). It is unclear if burnt spot is an early form of impoundment shell disease or if these two forms are unrelated (Cawthorn, 2011).

The third type of shell disease is epizootic shell disease, which has only been observed in the last 10 years from Southern Massachusetts to Eastern long Island Sound (Castro and Angell, 2000). Bacterial invasion begins on the surface of the dorsal cephalothorax where bacteria gain entry into the cuticle from tegumental gland canals, pore canals and sensory neuron canals (Kunkel et al., 2005; Smolowitz and Chistoserdov, 2005; Smolowitz et al., 2005). Flavobactericeae species are associated with epizootic shell disease but they do not infect lobster haemolymph or internal tissues (Chistoserdov et al., 2005; Quinn et al., 2013).

There are many challenges associated with studying the three forms of shell disease in lobsters as the initial abiotic, or biotic, factors that initiate shell disease are unknown. It is widely thought that shell disease results from both an opportunistic biotic agent and an environmental stressor. Temperature stress resulting from increased temperatures found in bays and shallows may predispose lobsters to shell disease (Smolowitz et al., 2005; Glenn and Pugh, 2006). It is widely believed that chitinolytic bacteria initiate shell

disease but it is unclear if they can infect a healthy carapace, or whether the disease may be caused by a malformation of the cuticle followed by a secondary bacterial infection (Tarsitano and Lavalli, 2005). To date, laboratory studies have been unable to transmit shell disease from infected to uninfected lobsters.

Epizootic shell disease prevalence is highest in May to June and lowest in August because lobsters normally moult in August, which results in a newly moulted shell free of lesions (Cawthorn, 2011). Shell disease is not isolated to certain lobster life stages and has been found in stage II larva, young of the year, juveniles and adults (Tlusty, 2005). Shell disease is highest in New England where prevalence greater than 50% have been found for ovigerous females in Eastern Long Island Sound (Landers, 2005) and from 20-30% in Southern New England (Castro & Angell, 2000, Cobb and Castro 2006).

1.3.4 Other Bacterial Diseases

Bacteraemic crustacean haemolymph is not uncommon (Rabin, 1965; Stewart et al., 1966; Sizemore et al., 1975; Tubiash et al., 1975; Bowser et al., 1981). Stewart et al. (1966) found bacteria in 24% of sampled lobsters but noted that this was probably an underestimate of the true prevalence due to the use of selective media. Additional culture-dependent (Chistoserdov et al., 2005; Bartlett et al., 2008) and culture-independent (Quinn et al., 2013) studies of lobster haemolymph have also found bacteria, but unlike *A. viridans*, most bacteria found in lobster haemolymph appear not to be pathogenic in low concentrations (Cornick and Stewart, 1968).

Species of *Vibrio* have been found in lobsters held at temperatures of 20 - 23 ^{o}C (Brinkley et al., 1976; Bowser et al., 1981) but these lobsters were undergoing temperature stress, and whether *Vibrio* spp. are primary or secondary pathogens of lobster is unclear. Limp lobster disease was reported in Maine in 1997 and 1998 but the causative agent is disputed as either *Vibrio fluvialis* (Tall et al., 2003) or *Hyphomicrobium (Photobacteria) indicum* (Giray and Bouchard, 2002). Both *V. fluvialis* and *P. indicium* are regarded as opportunistic pathogens of lobster but only when lobsters are significantly stressed (Robohm, 2005). Either way, the impact of these bacteria in wild populations is generally regarded as being underreported (Shields et al., 2006).

1.3.5 Viral Disease

There are currently no known naturally occurring viral pathogens of the genus *Homarus*. There is however a naturally occurring viral pathogen of the Caribbean spiny lobster *Panulirus argus* known as *Panulirus argus* virus 1 (PaV1) (Shields and Behringer, 2004). PaV1 is widely spread throughout the Caribbean (Butler el al., 2008; Huchin-Mian et al., 2009, Cruz Quintana et al., 2011) where prevalence is inversely proportional to lobster size (Shields & Behringer, 2004; Butler et al., 2008; Lozano-Álvarez et al., 2008). *Panulirus argus* virus 1 has been found to cause up to 100% mortality in experimentally infected juvenile *P. argus* (Shields and Behringer, 2004).

White Spot Syndrome Virus (WSSV) is the most significant factor inhibiting the growth and sustainability of global shrimp aquaculture (Stentiford et al., 2012). White Spot Syndrome Virus originated in Southeast Asia in the early 1990s but has now spread to

most shrimp aquaculture countries in Asia, the Americas, the Middle East and Europe (Walker and Mohan, 2009; Lightner and Redman, 2010; Sánchez-Paz, 2010; Stentiford and Lightner, 2011). The European Union has recently listed WSSV as capable of infecting all decapod crustaceans, including *H. americanus*, under Directive 2006/88/EC. White Spot Syndrome Virus has been found in experimentally infected *Panulirus versicolor* and *P. penicillatus* but these lobsters survived greater than 70 days without clinical manifestation of the disease (Chang et al., 1998). White Spot Syndrome Virus infected shrimp were fed to *P. versicolor, P. penicillatus, P. ornatus and P. longipes* and a polymerase chain reaction (PCR) based assay was able to detect low levels of the virus in all of the lobsters even though no clinical signs developed (Wang et al., 1998). Recent studies have found that two clawed lobsters, *H. gammarus* and *Nephrops norvegicus,* in the same Nephropidae family as *H. americanus* are susceptible to WSSV. White Spot Syndrome Virus is capable of infecting these lobster species through ingestion of WSSV-infected shrimp, or injection of WSSV-infected shrimp homogenate (Bateman et al., 2012a; 2012b).

The susceptibility to WSSV by *H. gammarus* and *N. norvegicus* points to the possibility that *H. americanus* could also be susceptible to WSSV infection, but there is currently no evidence that this is the case. Although viruses infect many crustaceans, including 20 in shrimp (Lightner and Redman, 1998) and 4 in blue crab (Shields et al., 2006), few have been well characterized outside of shrimp. As crustacean viral research continues, no doubt more viruses will be found.

1.4. Lobster Immune Response

The primary immune defense mechanism in lobsters is the shell or carapace, a continuous sheath of chitinous material, with a durable multilayer membranous covering at points of articulation, which is impervious to normal insults. However, some harvesting practices, and lobster-lobster or lobster-predator interactions, can cause integumentary damage which facilitates entry of pathogens into the lobster. Once inside the lobster, the potential pathogen faces numerous cellular and humoral innate defense mechanisms including: antimicrobial bactericidins, agglutinins, non-retracting clotting factors, phagocytosis by foreign-particle recognizing circulating and non-circulating cells, melanization as well as haemocyte-mediated encapsulation (Cornick and Stewart 1968; Acton et al., 1969; Stewart and Zwicker, 1972; Mori and Stewart, 1978; Goldenberg et al., 1984; Chisholm and Smith, 1995; Anderson and Beaven, 2005; DeGuise et al., 2005). Additionally, previous studies have demonstrated that pathogen infiltration activates several immune cascades (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004).

Any defect in the exoskeleton provides a portal of entry for opportunistic pathogens, results in rapid loss of haemolymph due to the open circulatory system of lobsters and disrupts osmotic integrity. Integumental defects can be caused by natural lobster-lobster and lobster-predator interactions, or inappropriate post-harvest handling practices (Stewart et al., 1969a; Stewart, 1980). Repair of these exoskeletal defects is initiated rapidly by the aggregation of haemocytes at the site of injury to minimize the potential detrimental aforementioned effects. Transglutaminase is released from the aggregated haemocytes to crosslink coagulogen, a clotting factor present in haemolymph analogous

to fibrinogen, thereby forming a clot (Doolittle and Fuller, 1972; Lorand, 1972; Lorand and Conrad, 1984; Martin et al., 1991). This clot is important to stop haemolymph loss and trap any opportunistic microorganisms and exogenous particles that may be present at the site of injury. The strength of the clot is directly proportional to the number of circulating haemocytes (Durliat and Vranckx, 1989). The marked haemocytopenia found in diseases such as gaffkemia and bumper-car disease causes significant delays in clotting times for infected lobsters, which can lead to exsanguination if the carapace is subsequently damaged (Rabin 1965; Rabin and Hughes, 1968; Stewart et al., 1968; 1969a; 1969b; Cornick and Stewart, 1968; Stewart and Zwicker, 1972; Cawthorn et al., 1996; Cawthorn, 1997; Athanassopoulou et al., 2004).

Clotting initiation promotes the migration and degranulation of haemocytes, which initiates the prophenoloxidation cascade to melanize the wound. Melanization causes the release of toxic metabolites in the immediate vicinity of the wound, thereby killing any immobilized opportunistic microorganisms (Nyhlen and Unestam, 1980; Söderhäll and Ajaxon, 1982). A new cuticle is formed beneath the melanin membrane by the involution of the epidermis into the wound using the haemocyte aggregation network for basal support (Fontaine, 1975).

Circulating granulocytic haemocytes and fixed phagocytes are very effective at identifying and phagocytosing foreign, or degraded lobster, particles found in the haemolymph. Fixed phagocytes are found in the terminal arterioles of the hepatopancreas

and are known to play a significant role in the elimination of foreign particles from the circulating haemolymph (Factor and Beekman, 1990; Johnson et al., 1981).

Circulating haemocytes are also responsible for recognizing and eliminating foreign particles that are too large to be phagocytized through haemocyte-mediated encapsulation (Hose et al., 1990). Haemocytes aggregate in response to cell damage or exogenous biotic or abiotic agents (Johnson, 1976; Newman and Feng, 1982) and this initiates the encapsulation of cellular debris or pathogens such as *A. haemophila*. Haemocyte aggregation is a synergistic reaction of both the cellular and humoral immune systems of the lobster. Large and small granular haemocytes are active in aggregation and release cellular aggregating proteins after the activation of the prophenoloxidase system.

Small and large-granule haemocytes are responsible for phagocytosis, while the hyaline haemocytes lyse in the presence of foreign particles, releasing clotting factors for encapsulation (Goldenberg et al., 1986; Hose et al., 1990). Large-granule haemocytes have a cytoplasm filled with many large-granules (> 1 μm diameter) and are recognized as a primary storage site for prophenoloxidase (Hose et al., 1990). Small-granule haemocytes have granules < 1 μm and contain lysosomal enzymes. It is the small-granule haemocytes that are the primary haemolymph circulating cell involved in phagocytosis (Hose et al., 1990). The third type of haemocytes are hyalinocytes, which have many small electron dense deposits in their cytoplasm. They have a variable number of small-granules (< 1μm) and their primary role is in haemolymph coagulation. *Homarus americanus* haemocytes have been extensively characterized where 11 different

haemocytes have been classified although several are considered precursors of the aforementioned haemocytes cell types (Battison et al., 2003). Transglutaminase, the essential enzyme in haemolymph clotting, is present in higher concentrations in haemocyte fractions with greater numbers of hyaline cells (Martin et al., 1991).

Lobsters also have a strong humoral immune system based on a variety of haemolymph proteins which are capable of recognizing foreign particles namely: agglutinins, antimicrobial peptides and opsonins. Lobster agglutinins are protein molecules capable of recognizing carbohydrate moieties present on the extracellular surface of pathogenic organisms (Cornick and Stewart, 1973; 1978). Characteristic pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), 1-3-glucans or peptidoglycans are bound by pattern-recognition receptors (PRR) present in the haemolymph, which are serine protease homologues (Lee and Söderhäll, 2001). The PAMP-PRR complexes can bind to granular haemocyte receptors, which causes them to degranulate and release prophenoloxidase (Söderhäll et al., 1996; Sritunyalucksana and Söderhäll, 2000). Prophenoloxidase is activated by cleavage to phenoloxidase by a serine protease known as prophenoloxidase-activating enzyme. Phenoloxidase then catalyzes the conversion of phenols in the haemolymph to generate quinones that polymerize to form melanin and toxic metabolites like reactive oxygen species (Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1998).

Antimicrobial peptides produced by lobsters are small (~10 kDa) proteins integral to the innate immune system of all prokaryotes, animals and plants (Lehrer and Ganz, 1999;

Bulet et al., 1999; Reddy et al., 2004). Lobster antimicrobial activity has been found to be greatest in lobster serum (Acton et al., 1969; Stewart and Zwicker, 1972; Mori and Stewart, 1978). Three lobster antimicrobial peptides, Hoa-crustin, CAP-1 and CAP-2, have recently been identified and characterized (Christie et al., 2007; Battison et al., 2008). It is likely that these antimicrobial peptides have opsonization as well as direct antimicrobial activity.

Another family of antimicrobial peptides is the anti-lipopolysaccharide factors (ALF). These are basic proteins approximately 100 amino acids in length that were initially isolated in the haemolymph of horseshoe crabs (Aketagawa et al., 1986). Although initially believed to act only on Gram-negative LPS containing bacteria (Chaby, 2004), they have also been shown to be active against Gram-positive bacteria (Imjongjirak et al., 2007) and their gene-expression is upregulated in response to WSSV infections (Liu et al., 2006; 2011; Robalino et al., 2007; Tharntada et al., 2009; Leu et al., 2013). Multiple ALF isoforms have been described in shrimp (Supungul et al. 2004; Tassanakajon et al. 2006; Tharntada et al. 2008), and two isoforms have been described for *H. americanus* (Beale et al., 2008). The ALF isoforms isolated from *H. americanus* (ALFHa), known as ALFHa-1 and ALFHa-2, were shown to be differentially expressed in response to an injection challenge experiment with *V. fluvialis*. No change in ALFHa-2 expression was found in hepatopancreas, gill or hematopoietic tissues while ALFHa-1 expression was upregulated 17-fold in hepatopancreatic tissue, but not in gill or hematopoietic tissue (Beale et al., 2008). The number of ALF isoforms in shrimp, along with the differential

expression of the ALF isoforms in *H. americanus*, suggests there may be some substrate specificity at least at the pathogen class level, but this has not been explored to date.

Lobsters are capable of producing lectins and α_2-macrogobulin (Spychert et al., 1987; Battison and Summerfield, 2009). Invertebrate lectins are selective carbohydrate-binding proteins capable of acting as agglutinins, opsonins and occasionally antimicrobial peptides (Marques and Barracco, 2000; Young Lee and Söderhäll, 2002; Quesenberry et al., 2003) and are a key part of the self and non-self recognition system. The first lobster lectins were identified by Hall and Rowlands (1974a) who further characterized them as agglutinin I and II (Cornick and Stewart 1973, Hall and Rowlands 1974b). N-acetyl-galactosamine and sialic acid residue-specific lectins have also been identified in *H. americanus* (Durliat and Vranckx, 1989). Recently, Battison and Summerfield (2009) have identified four additional novel lobster lectins. A 342 kDa lobster α_2-macroglobulin protein has also been identified that inhibits a wide variety of endoproteases, although its specific role in the humoral immune response remains unknown (Martin and Hose 1995).

1.5 High-throughput Immunological Effector Molecule Discovery

Studies that fixate on the function or expression of "classical" immune genes limit our understanding of immunology to what is already known, and narrow our frame of reference to overly simplified immune paradigms. This is especially detrimental when studying non-traditional animal models, such as lobster, where little is known about the specificity and scope of its immune responses. As we broaden our focus to include as

many genes as possible, we remove our traditional vertebrate immune gene biases and allow the discovery of novel crustacean immune molecules, or genes, with additional immune-related function. This discovery driven approach is ideally suited to high-throughput genomic and transcriptomic techniques which offer an excellent complementation to hypothesis-driven experimentation.

Gene expression microarrays and related techniques facilitate the discovery of the transcriptome, which has the potential to unveil a plethora of traditional and novel immune molecules or immune signaling pathways (Heng and Painter, 2008). A recent review of the crustacean immune system (Hauton, 2012) stated that the limited genomic sequence data for commercially important crustacean species, such as *H. americanus*, has posed a significant impediment to crustacean immunological research. Microarray and other high-throughput sequencing technologies have been called upon to overcome these research limitations and examine the breadth and specificity of the crustacean immune system (Hauton, 2012).

The ambitious Immunological Genome Project has embarked on an effort to complete a 'road map' of gene-expression in murine immune cells. This project has chosen to use a microarray platform for gene discovery as the transcriptome is the only 'ome' that can be elucidated in its entirety (Heng and Painter, 2008). High-throughput molecular characterization of gene expression has become an important approach to elucidate crustacean immunology in a rapid and cost-effective manner. This microarray approach has been applied to shrimp stress (de la Vega et al., 2007), reproduction (Karoonuthaisiri

et al., 2009; Leelatanawit et al., 2011; Brady et al., 2013) and immune response (Dhar et al., 2003; Wang et al., 2006; 2008; Robalino et al., 2007; Wongpanya et al., 2007; Aoki et al., 2011; Pongsomboon et al., 2011; Veloso et al., 2011; Fagutao et al., 2012). An extensive expressed sequence tag (EST) library for several *H. americanus* tissues has been sequenced and uploaded into GenBank (Towle and Greenwood unpublished). This library facilitated the generation of a 15,376 feature *H. americanus* microarray representing over 14,000 genes. This novel microarray tool represents the first high-throughput gene expression analysis tool for monitoring changes in *H. americanus* gene expression to countless physiological conditions including response to pathogenic pressures.

1.6 Rationale for study

There is currently a paucity of information regarding the immune system in commercially valuable lobster species. The application of a high-throughput gene expression analysis tool like the novel microarray developed by Towle and Greenwood (unpublished) has the potential to greatly expand our understanding of disease, disease susceptibility and host immune response in *H. americanus*. The discovery of the genetic origin of upregulated immune factors could provide the basis of diagnostic biomarkers for lobster disease and health status. These biomarkers can be used to: protect live-lobster holding facilities from catastrophic losses due to disease, investigate natural mortality events, epidemiological surveys of temporal and geographic disease prevalence, and to understand the impact of anthropogenic effects on marine ecosystems due to pesticides, agricultural runoff, heavy metal contamination or the effects of climate change and ocean acidification.

In addition to being a commercially valuable species, lobsters offer many advantages as experimental models of crustacean immunity. Lobsters are inexpensive, plentiful, relatively large at legal harvest (>400g), easy to maintain for long periods of time and it is easy to obtain large haemolymph samples for downstream analysis. Research to date has primarily focused on haemolymph macromolecular and histopathologically evident mechanisms of immunity, but this study aims to elucidate the differential expression behind the genetic basis of immunity in adult lobsters using high-throughput gene expression analysis. This high-throughput methodology has the potential to rapidly expand our knowledge of lobster and crustacean immunity. I hypothesize that:

***Homarus americanus* infected with bacterial, parasitic or viral pathogens will result in quantifiable differential expression of immune-related genes.**

The objectives of this study are:

1. Conduct an *Aerococcus viridans* var. *homari in vivo* challenge in *H. americanus.*
    a. Verify *A. viridans* infection clinically and histologically.
    b. Monitor hepatopancreatic global gene expression using a *H. americanus*-specific microarray.
    c. Verify the microarray gene expression findings using RT-qPCR.

2. Conduct an *Anophryoides haemophila in vivo* challenge in *H. americanus*,
    a. Verify *A. haemophila* infection clinically and histologically.
    b. Monitor hepatopancreatic global gene expression using a *H. americanus*-specific microarray.

c. Verify the microarray gene expression findings using RT-qPCR.

3. Conduct White Spot Syndrome Virus *in vivo* challenge in *H. americanus*,
    a. Verify WSSV infection clinically, histologically and with qPCR.
    b. Monitor hepatopancreatic global gene expression using a *H. americanus*-specific microarray.
    c. Verify the microarray gene expression findings using RT-qPCR.

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2. Differential expression of American lobster (*Homarus americanus*) immune related genes during infection of *Aerococcus viridans* var. *homari*, the causative agent of Gaffkemia

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2.1 Abstract

This is the first transcriptomic study focusing on immunity in the commercially valuable American lobster (*Homarus americanus*). We have conducted an *in vivo* infection trial using the Gram-positive bacterium *Aerococcus viridans* var. *homari* to determine how *H. americanus* responds to this naturally occurring lethal-pathogen. A novel *H. americanus* microarray was used to measure the transcriptomic changes occurring in over 14,000 genes in the lobster hepatopancreas. Hundreds of new immune genes and isoforms were identified and measured for the first time in this species, and our findings highlight 148 genes of interest involved in *H. americanus* pathogen response. We verified our microarray results using RT-qPCR on three anti-lipopolysaccharide (ALFHa-1, ALFHa-2, ALFHa-4), a thioredoxin, acute phase serum amyloid protein A, hexokinase and two trypsin genes. RT-qPCR and microarray findings show close agreement and highlight the significant increase in gene expression in many lobster immune genes during *A. viridans* infection. Differential expression of the ALFHa isoforms may indicate that the *H. americanus* immune response can be tailored to the class of pathogen causing disease.

2.2 Introduction

The American lobster (*Homarus americanus*) fishery is the most economically significant commercial fishery in Atlantic Canada with annual values of approximately $CAN 1 billion (Gardner Pinfold, 2010). The adult lobster is relatively resistant to a wide variety of opportunistic marine pathogens, however, there are several of concern including *Aerococcus viridans* var. *homari* (Cawthorn, 2011). *Aerococcus viridans* is a Gram-positive bacterium that causes a lethal disease in *H. americanus* and has been found at a prevalence of between 1% and 32% in the natural marine environment (Rabin, 1965; Stewart et al., 1966; Lavallée et al., 2001). However, little is known about how the lobster immune system responds to this, or any other, pathogen.

Recently, two antimicrobial peptides and four plasma lectins have been described for *H. americanus*, and may be involved in the immune response (Battison et al., 2008; Battison and Summerfield, 2009). These plasma lectins belong to a diverse array of pattern recognition proteins (PRPs) which have been recognized as key sensors of pathogen infection which initiate the crustacean immune response (Hauton, 2012). There has also been two anti-lipopolysaccharide factors identified for *H. americanus* (ALFHa-1 and ALFHa-2), where ALFHa-2 has been shown to be upregulated in gill tissue during *Vibrio fluvialis* infection (Beale et al., 2008). A crustin antimicrobial peptide found in an EST database, but not tested for its biological functionality, has also been described for *H. americanus* (Christie et al., 2007). These crustins and ALF antimicrobial proteins are common in many crustaceans where they have been found to have activity, or become

transcriptionally active, during a wide variety of bacterial, protozoal, fungal and viral infections (Hauton, 2012). There have also been a few haemolymph-based agglutinin factors studied but only rudimentary information on host response was presented with nothing on protein or nucleotide sequence identification (Cornick and Stewart, 1973; Hall and Rowlands, 1974a; 1974b; Hartman et al., 1978; Abel et al., 1984).

The limited information available on *H. americanus* immune factors presents an excellent opportunity for a high-throughput genomic approach to reveal genetic mediators of the lobster's immunological response. A recent review of crustacean immunity (Hauton, 2012) has highlighted that the lack of annotated EST and genomic sequence data as an impediment to the rate and scope of crustacean immune factor discovery. Hauton (2012) further postulated that annotated genomic sequence, coupled with molecular techniques such as microarrays, would significantly advance our understanding of crustacean immunity.

The purpose of our study was to investigate the *H. americanus* immune system by monitoring the transcriptomic changes that occur during infection with a natural marine pathogenic bacterium *A. viridans* var. *homari*. We used a novel *H. americanus* microarray to monitor the expression of 14,592 hepatopancreatic genes. Our goal was to discover new immune genes or isoforms involved in the lobster's immune system. These findings illustrate that the use of high-throughput molecular techniques to investigate *H. americanus* immunity marks a significant advance in our understanding of how lobsters respond to disease.

2.3 Experimental Procedures

2.3.1 Acclimation and Animal Handling

Adult male American lobsters, *H. americanus* (n = 27, 570 g ± 11.0 g) were purchased from a local seafood supplier (Bedford, Nova Scotia) and assessed for physical and haemolymph quality as well as pathogen-free status. All lobsters were given an overall health assessment and screened for pathogens (Acorn et al., 2011). Only healthy, pathogen-free lobsters were selected for inclusion in the study. Lobsters were individually housed in a recirculating artificial seawater (ASW) (Instant Ocean, 30 ppt) system equipped with UV filtration (Aquabiotech, Coaticook, QC, Canada) in the Aquatic Animal Facility of the Atlantic Veterinary College in accordance with the University of Prince Edward Island Animal Care Committee approved animal care protocol #09-052. Lobsters were acclimated to 15 °C by increasing the water temperature from 5.5 °C by 1 °C per day to mimic the natural water temperature where *A. viridans* becomes especially lethal. Lobsters were held for 2 weeks prior to the beginning of the experiment. Lobsters were not fed during this experiment.

2.3.2 Bacterial Strains

A virulent *A. viridans* var. *homari* strain (Rabin's) was used in the *in vivo* infection experiment (Greenwood et al., 2005). Bacterial cells were cultured in 250 mL Erlenmeyer flasks containing 50 mL of sterile Tryptic Soy Broth at 28 °C for 18 - 24 h, prior to collection by centrifugation at 2000g for 5 min. Bacterial pellets were then washed twice

in sterile 3% NaCl, prior to resuspension in 3% NaCl to achieve 7.5×10^7 colony forming units (CFU) per mL as determined by optical density measurement.

2.3.3 Bacterial Infection Challenge

Lobsters were randomly assigned to receive either 200 µL injections of *A. viridans* (n = 20) or sterile 3% NaCl (n = 7). Bacterially challenged lobsters (n = 4) were randomly selected for lethal sampling at 6 h, 12 h, 24 h, 48 h post-injection (pi) and when the lobsters became moribund at 78 h pi. Lobsters which had received the sterile 3% NaCl injections were removed at 6 h pi (n = 3) and 78 h pi (n = 4).

2.3.4 *H. americanus* Sampling

Lobsters were lethally sampled by severing the ventral nerve cord between the first set of periopods and the chelipeds to obtain *A. viridans* haemolymph concentration information, as well as tissues for histologic and RNA analysis. Hepatopancreas, heart, gill, antennal gland, testis, stomach, intestine, tail muscle and claw muscle were preserved for histological examination. Tissues for histology were preserved in 10% formalin in 0.2 µm filtered ASW for a minimum of 7 days. Samples were dehydrated and infused with paraffin wax using an infiltration processor and standard protocols. A rotary microtome was used to cut 3 - 5µm serial sections which were then mounted on glass slides and stained with haematoxylin and eosin (H&E). Stained sections were evaluated using a light microscope (Zeiss Axioplan 2).

Lobster hepatopancreatic tissue was preserved for microarray and RT-qPCR analysis by immediate homogenization with an OMNI homogenizer (OMNI International, Kennesaw, GA, USA) in RNA preservation reagent (1.4 M guanidine isothiocyanate, 38% phenol (pH 4), 5% glycerol and 0.1 M sodium acetate) at a ratio of less than 100 mg tissue/mL RNA preservation reagent. Homogenized tissue was then flash frozen in liquid nitrogen and stored at -80 °C until use.

Haemolymph was serially diluted in 3% NaCl and 6 × 25 µL of diluted haemolymph was spotted on Blood Agar plates supplemented with 3 % NaCl. Plates were incubated at 37 °C and the number of colonies was counted after 48 h.

2.3.5 RNA Extraction

RNA was obtained from thawed hepatopancreatic tissues previously homogenized in RNA preservation reagent. Briefly, 200 µL of chloroform per mL of RNA preservation reagent was added to thawed tissue homogenate. The solution was shaken for 10 s and left at room temperature for at least 3 min and centrifuged at 12,000*g* for 15 min at 4 °C. The resulting supernatant was removed and 600 µL was added to 600 µL of 70% ethanol. The RNA was extracted with a RNeasy spin column (Qiagen, Toronto, ON, Canada) using the manufacturer's instructions including the optional DNase I (Qiagen) treatment. RNA was quantified using spectrophotometry (NanoDrop ND-1000, Thermo Fisher, Ottawa, ON, Canada) and a quality assessment was performed using an Experion microcapillary electrophoresis unit (BioRad, Mississauga, ON, Canada) and stored at -80 °C until use.

2.3.6 *H. americanus* Microarray Design and Construction

Arrays were designed based on publically available gene sequences and ESTs determined from cDNA libraries representing multiple tissues of male and female lobsters, of various physiological conditions (Towle and Greenwood, unpublished). In total, 29,636 ESTs for the *H. americanus* were available in dbEST (www.ncbi.nlm.nih.gov/genbank). Unique ESTs were determined using CLOBB and Gene Indices Clustering Software (TIGR) (Parkinson et al., 2002; Pertea et al., 2003). The final outcome of the two clustering methods was 15,864 unique sequences. To construct the *H. americanus* array, 50 mer probes were designed using Array Designed 4 software (Premier Biosoft International), producing 14,592 probes of high binding specificity, GC content, and annealing temperature (Towle and Greenwood, unpublished). The probes were synthesized by Integrated DNA technologies (Coralville, IA) (IDT) and submitted to the Vancouver Prostate Centre DNA microarray facility in 250 µM aqueous solutions. Oligonucleotides were printed on Erie C28 Aminosaline coated glass slides (2 arrays/slide) using a QArraymax arrayer. The array included 14,592 lobster sequences, 210 Sigma Alien DNA controls, 78 GFP controls, 80 GFP landmarks and 416 buffer controls, a total of 15,376 spots. Printing quality was monitored using a 9 mer hybridization GenePix 4200AL scanner and visualized with Imagene version 8.0.1.

2.3.7 cDNA Labeling and Microarray Hybridization

High quality sample RNA was used to generate fluorescently Alexa Fluor® 555 labeled single strand cDNA using 20 µg of RNA and SuperScript™ Plus Indirect cDNA Labeling

System (Life Technologies Inc., Burlington, ON, Canada). In addition, reference aRNA was generated from 5 µg of pooled lobster hepatopancreatic RNA using an Amino Allyl MessageAmp™ II aRNA Amplification kit (Life Technologies Inc.). Reference aRNA was Alexa Fluor® 647 labeled using 12 µg of aRNA and a Superscript™ Plus Indirect cDNA Labeling System (Life Technologies Inc.). Labeled aRNA and cDNA were quantified spectrophotometrically (NanoDrop ND-1000) and 140 ng of cDNA and 100 pmol of labeled aRNA were used for microarray hybridization. Prior to hybridization, labeled aRNA was fragmented using 1 µL 10× Fragmentation solution and incubation for 10 min at 70 °C followed by 1 µL of Stop solution (Life Technologies Inc.). Hybridizations were performed using A2 chambers on a Tecan 400 HS Pro hybridization system (Tecan, NC, USA). Briefly, microarrays were incubated at 65 °C for 10 min, washed twice with hybridization buffer 1 (5× SSC, 0.01% SDS, 0.2% BSA) for 20 s at room temperature, incubated at 50 °C for 13 min, washed once with buffer 1 for 20 s, incubated at 48 °C for 13 min, washed once with buffer 1 for 1 min and once with buffer 4 (5× SSC) for 1 min. The sample was then injected and hybridized to the microarray with sample agitation at 48 °C for 16 h. The microarray was washed with buffer 2 (2× SSC, 0.2% SDS) at 40 °C for 1 min and incubated at 40 °C for 2 min, washed with buffer 2 at 30 °C for 1 min and with buffer 5 at 30 °C for 1 min, incubated at 30 °C for 2 min, washed with buffer 5 at 23 °C for 1 min, and buffer 3 (0.2× SSC) at 23 °C for 1 min. Finally the microarray was incubated at 23 °C for 2 min followed by a wash with buffer 3 for 1 min and then dried with nitrogen gas for 3 min.

2.3.8 Microarray Data Analysis

Microarrays were imaged using a GenePix 4000B scanner (Molecular Devices, PA, USA) and features were extracted using SpotReader v 1.3.1 (Niles Scientific, CA, USA). Data was analyzed using TM4/MeV v4.8.1 (Saeed et al., 2003). Two color gene expression arrays were normalized by LOWESS and flagged using the MIDAS (Microarray Data Analysis Software) v2.2.2 software (Quackenbush, 2002). The data was used to generate log2 ratios of sample to reference gene intensity so that all arrays could be compared. One way ANOVA analysis (MeV) was used at $\alpha = 0.05$ with and without Bonferroni correction and at $\alpha = 0.005$ without Bonferroni correction. Blast2GO (Conesa et al., 2005) was used to find similarity between the *H. americanus* ESTs used in this study and proteins in GenBank. Figure of Merit (FOM) and K-means clustering were used to determine similarity between the expression profiles of significantly differentially expressed genes (Soukas et al., 2000; Yeung et al., 2001).

2.3.9 Quantitative Reverse Transcriptase PCR (RT-qPCR)

Microarray results were verified by measuring the mRNA expression of eight selected genes of interest by RT-qPCR. Normalization genes were validated using geNorm^{PLUS} in the qbase^{PLUS} qPCR analysis software suite (Biogazelle, Belgium). A total of 2 genes (CHP and Hyploc) were determined to be the minimal number of normalization candidates needed based on them having an average geNorm^{PLUS} M value of 0.245 and a combined geNorm^{PLUS} V value of 0.085. cDNA was generated using 1 µg of sample RNA with the Superscript III First Strand Synthesis kit (Life Technologies Inc.), with oligo d(T) primers as per the manufacturer's instructions. qPCR reactions were performed

using 2 µL of cDNA (1/60 dilution) in a total reaction volume of 15 µL using a Chromo4 Real-Time PCR system (BioRad, Hercules, CA). Each qPCR reaction contained 1× Express SYBR GreenER with ROX (Life Technologies Inc.) and 200 nM of each primer. Primer sequences are shown in Table 2.1 and reactions were not optimized for primer concentration because reaction efficiencies were acceptable and incorporated into the RT-qPCR analysis.

Both two-step and three-step qPCR reactions were used in order to meet reaction efficiency and reproducibility requirements. Two-step qPCR protocols were as follows: 50 °C for 2 min 95 °C for 2 min followed by 39 cycles of 95 °C for 7 s then 20 s at the gene-specific annealing temperature followed by a plate read. Three-step qPCR protocols were as follows: 50 °C for 2 min 95 °C for 2 min followed by 39 cycles of 95 °C for 7 s then 20 s at the gene-specific annealing temperature and 72 °C for 20 s followed by a plate read. Melt curve analysis was then performed to determine product specificity over the temperature range of 65 – 90 °C in 1 °C increments every 2 s. Amplicons were analyzed on 2% agarose gels to confirm product size. Each sample was run in triplicate for qPCR analysis and every run contained a negative RT and negative template control to confirm the absence of contaminating genomic DNA.

2.4. Results

2.4.1 Confirmation of Disease Model

The disease model was confirmed to be following the natural pathology in *H. americanus* by monitoring the numbers of circulating bacteria in the haemolymph and histological analysis. Nine *H. americanus* tissues were examined histologically: hepatopancreas,

antennal gland, gill, heart, claw muscle, gut, tail muscle, testis and stomach. All tissues displayed typical pathology associated with *A. viridans* infection. Bacteria initially concentrated in the fixed phagocytes of the hepatopancreas (Fig. 2.1) and were observed in smaller numbers in the gill and antennal gland tissues. Over the course of the infection trial, the bacterial infection became systemic and *A. viridans* were seen in all tissues of infected lobsters by 76 h pi. Exponential growth of bacteria was observed with increases of over 5-fold in the first 24 h and 48 h and over 25-fold in the last 24 h. Final bacterial loads reached an average of 9.48×10^8 CFU/mL by the end of the trial (Fig. 2.2).

2.4.2 Analysis of Differential Gene Expression

Lobster hepatopancreatic tissue gene expression was measured with a *H. americanus* microarray during bacterial infection. Several approaches were used in conjunction with Hierarchical Clustering (HC) to determine which analysis would provide the most accurate and biologically relevant genes that were significantly differentially expressed. After significance analysis, 927 differentially expressed genes were found at $\alpha = 0.05$ and 24 genes after a Bonferroni correction was applied. Hierarchical Clustering on the 24 Bonferroni corrected genes indicated that samples could be loosely separated into groups: controls and 6 h pi infected, 12 h and 24 h pi infected and then 48 h and 76 pi infected animals (Fig. 2.3). A one-way ANOVA performed at $\alpha = 0.005$ resulted in 148 genes meeting the significance criteria (Appendix A Table A.1). Hierarchical Clustering on these 148 significant genes was able to differentiate the 6 h and 76 h pi controls from the infected samples. The infected samples separated such that 75% of the 76 h and 50% of

Table 2.1 Forward and reverse primers used in RT-qPCR experiments.

Gene	Accession #	Forward	Reverse	Annealing Temperature (°C)	Reaction Efficiency	# Step Reaction
ALFHa-1	EU625516	CAGTCGTTCTGGTGTTGTTGGGAA	TTGTTGGGCATCCCTCTCGGTTAT	65	99	3
ALFHa-2	FC556430	AGACTACCACTGACTTCGTGAGGA	TCTCGGGATGATCCGTTAACACCT	65	95	3
ALFHa-4	DV772634	ACAAGACAAGAGAATGCGTCCCTC	TGATAGCTTGTCCACGAAGGCTG	65	93	3
Thioredoxin	FD699182	TTTGACAAGCAGTTGGCTGATGCC	TCCACGTCCACCTTCAAGAACACT	65	93	3
SAA	EH116055	TACCACTACCAGCACTCATCACCT	TCAAACACAGAGAATAGGCACGGG	59	99	3
Hexokinase	EH401720	TGAACATTCTGGAGAGATGCCGGA	GGAGTCTTTGGCCAAGCCTTTGTT	65.2	100	2
Trypsin 1a	EV781656	CGTCCAATGTTAAGCGTCATGCCA	TTCCAAGTCTTGCCCGTAGACACA	65	93	3
Trypsin 1b	EF095144	AGCTACCGCAACATCGGCTATACT	ATGAAGTAGCGGTTGTCAGCTCCA	67	92	3
CHP	FE659358	TCAAGCCTGAAGCTGGGATATGCT	AAACACATGGGTTGGATGGCACAG	66.5	92	2
Hyploc	FE535262	ACATGGCAGTGGAAGACTCAAGGA	AAAGGAAACTGCGAACACTGCTGG	62.5	89	2

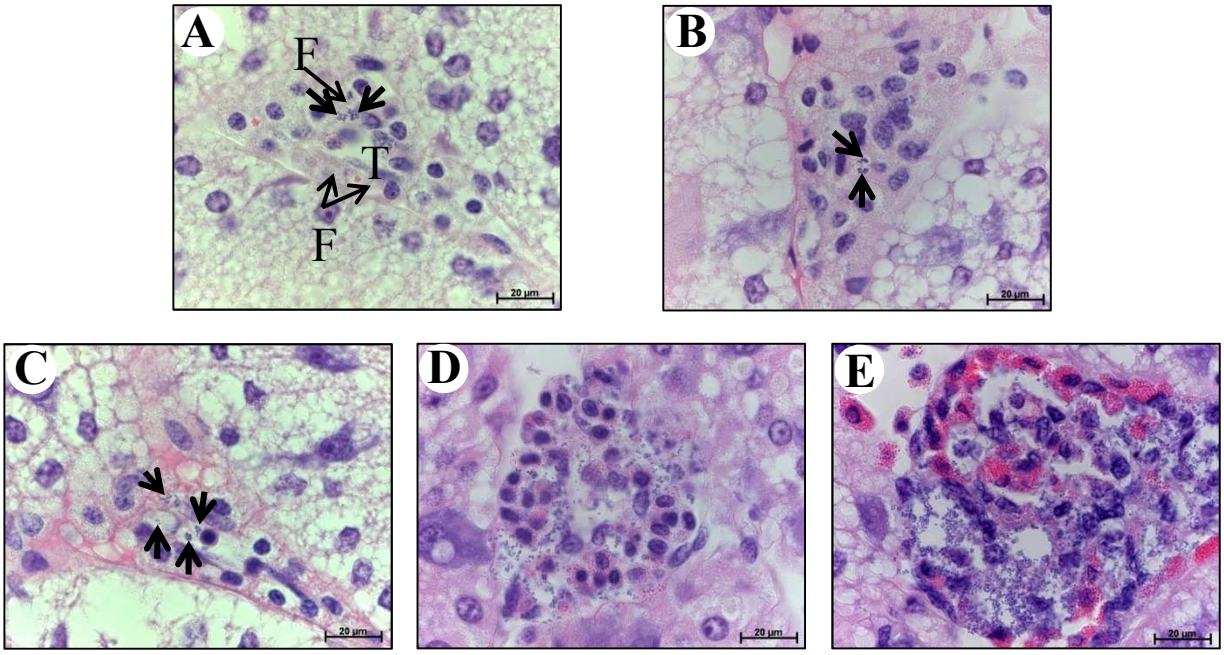

Figure 2.1. Histopathology of *Homarus americanus* hepatopancreas after *Aerococcus viridans* var. *homar*i infection. A) 6 h, (B) 12 h, (C) 24 h, (D) 48 h and (E) 76 h. Bold arrows indicate bacterial coccus tetrads and clusters located in fixed phagocytes (F) surrounding a terminal arteriol (T). Haematoxylin and eosin staining.

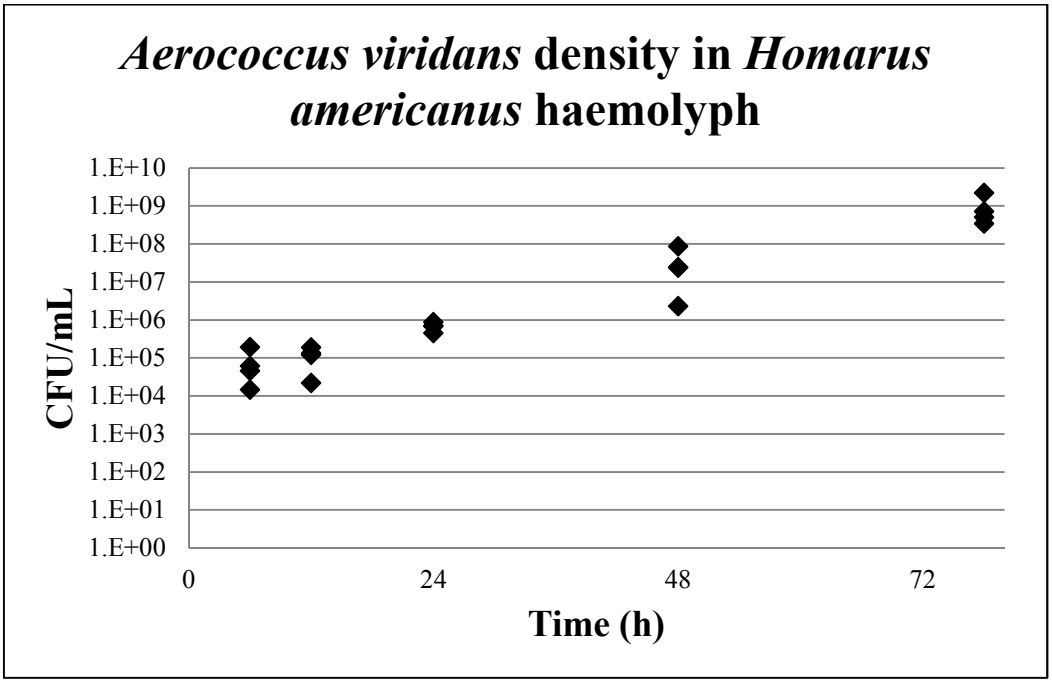

Figure 2.2 *Aerococcus viridans* density in *Homarus americanus* haemolymph during bacterial challenge (n = 4 at each time point).

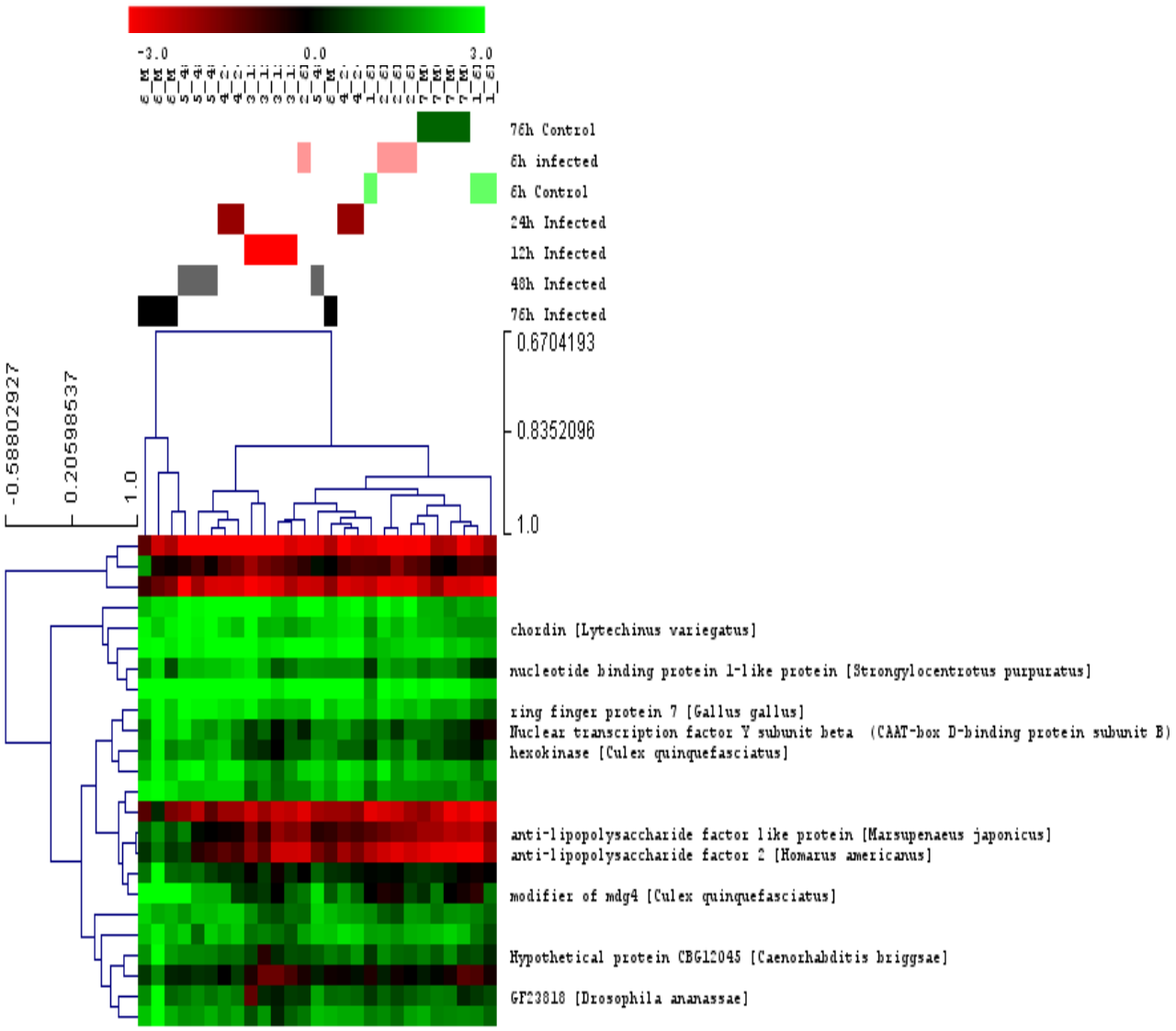

Figure 2.3 Hierarchical Clustering of expressed genes from *Homarus americanus* following *Aerococcus viridans* var. *homari* infections. 24 Bonferroni corrected significantly differentially expressed genes. Gene expression heat maps illustrate the ratio of gene expression of an experimental sample to the reference sample, where a gradient of red to green represents a three-fold or greater decrease in gene expression to a three-fold or greater increase in gene expression.

the 48 h pi infected samples separated from the remainder of the infected samples, while 100% of the 24 h, 75%samples separated from the remainder of the infected samples, while all of the 24 h, 75% of the 6 h 50% of the 48 h and one of the 76 h pi infected animals separate from the 12 h pi infected samples (Fig. 2.4).

Blast2GO was used to assign functional information to the 148 genes significant at $\alpha = 0.005$ by adding Gene Ontology (GO) information. The BLASTx feature of Blast2GO found similarity between lobster genes and proteins in the NCBI database for 51% of the significant ESTs, where 46.9% had corresponding GO information (Fig. 2.5). Ontology descriptions were provided in the cellular component, molecular function and biological process categories at level 3.

The 148 significantly differentially expressed genes were separated into groups based on similar expression patterns. The microarray analysis values were averaged for each treatment time point and the Figure of Merit (FOM) algorithm was used to calculate that four clusters would be the minimum number of K-means clusters that will generate clusters with strong predictive power (data not shown). K-means clustering allowed the differentially expressed genes to be clustered into four groups, each following four distinct patterns of expression during our infection trial (Fig. 2.6 and Appendix A Table A.1). Cluster 1 contains 16 genes that were downregulated during infection, Cluster 2 contain 53 genes that were upregulated during infection. Cluster 3 contains 31 genes that were upregulated but displayed a biphasic pattern with an increase at 6 h, a decrease at 12 h and an increase at 48 h of infection. Finally, cluster 4 contains 48 genes and was

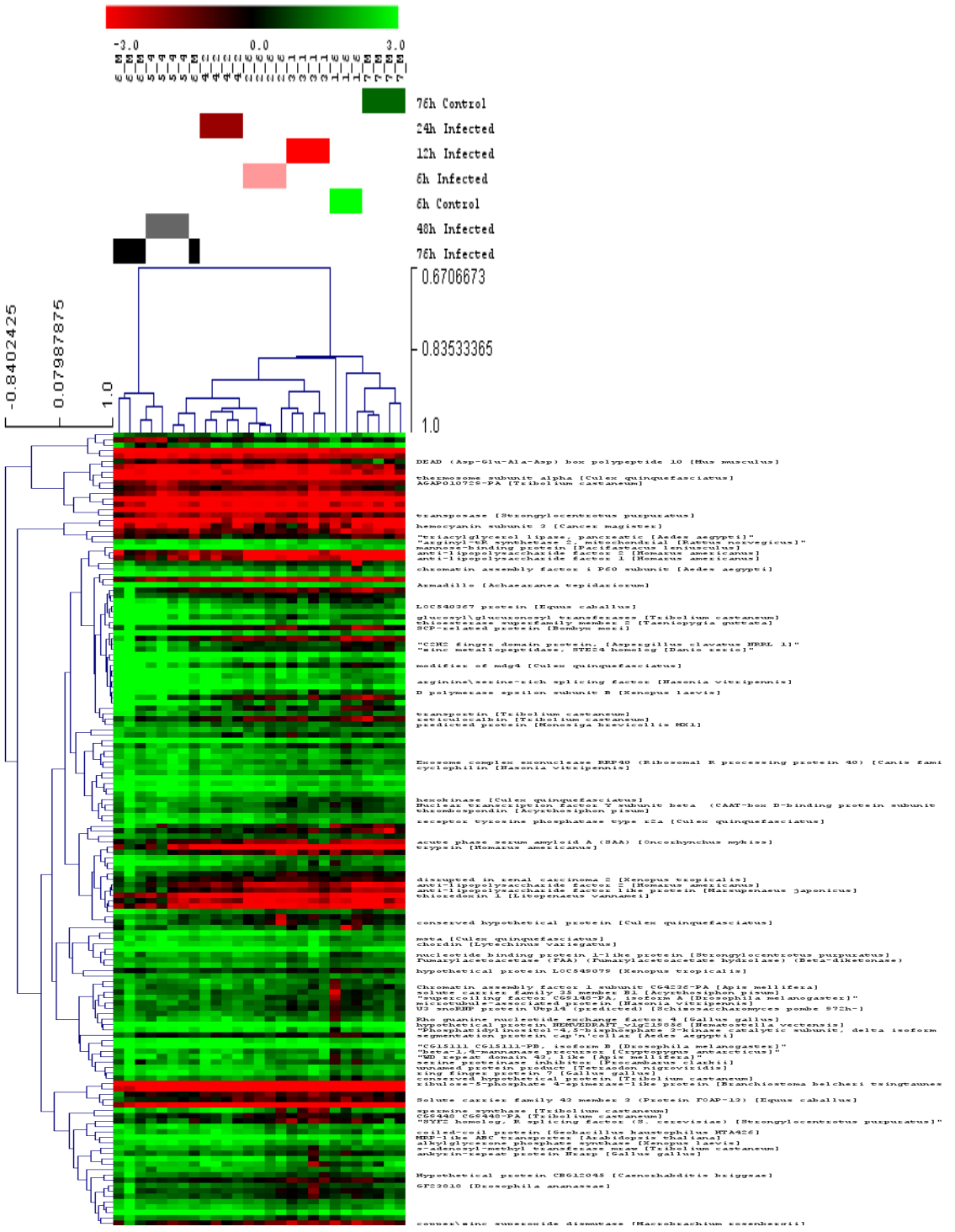

Figure 2.4 Hierarchical Clustering of expressed genes from *Homarus americanus* following *Aerococcus viridans* var. *homari* infections. 148 differentially expressed genes at a significance of $\alpha = 0.005$. Gene expression heat maps illustrate the ratio of gene expression of an experimental sample to the reference sample, where a gradient of red to green represents a three-fold or greater decrease in gene expression to a three-fold or greater increase in gene expression.

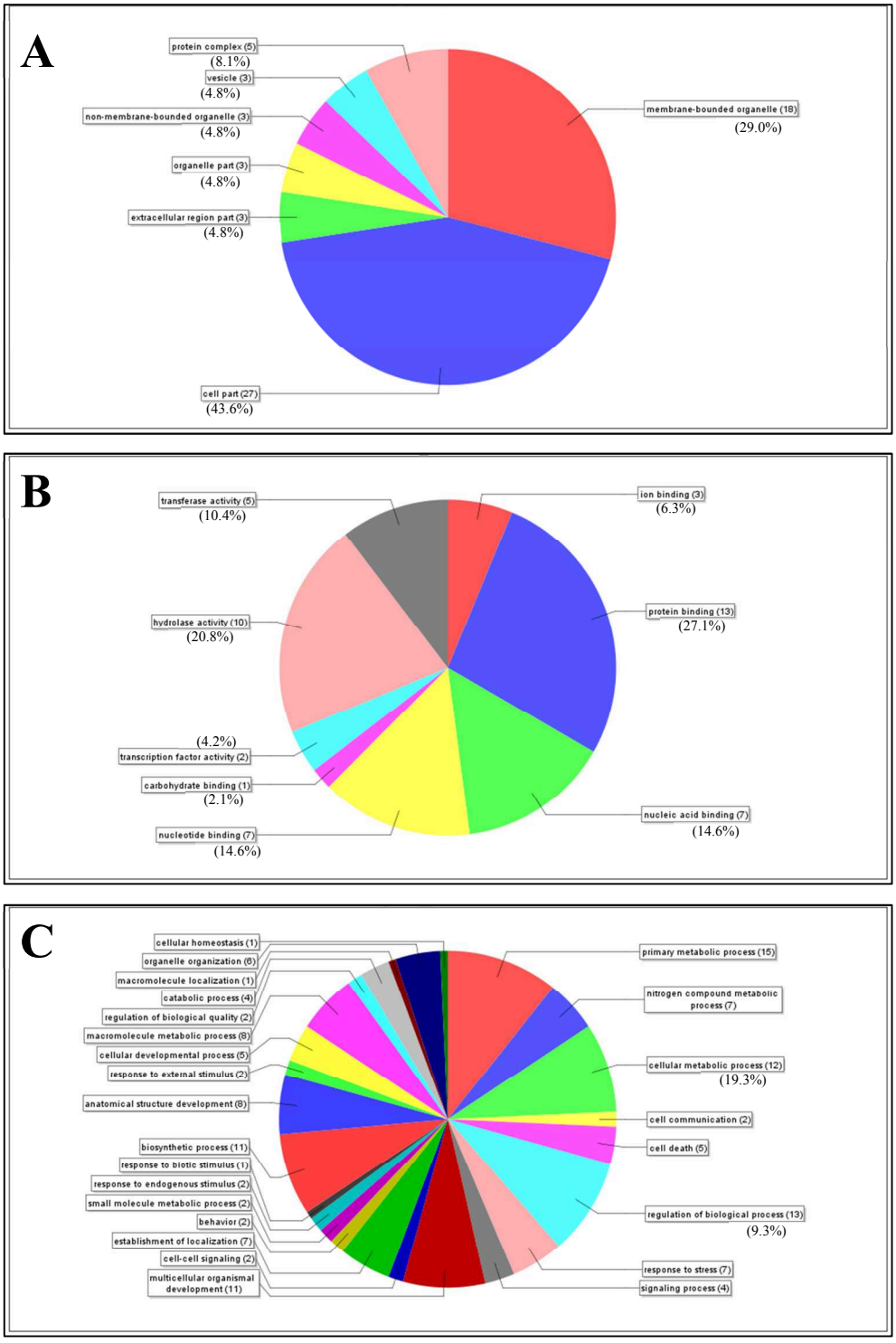

Figure 2.5 Gene ontology information for the 148 significantly different *H. americanus* expressed genes as assigned by BLAST2GO at level 3. (A) Cellular component, (B) Molecular function and (C) Biological process.

similar to cluster 3 where the drop in expression at 12 h found in cluster 3 was less pronounced (Fig. 2.6).

2.4.3 Verification of Differential Gene Expression Via RT-qPCR

Several genes were selected from the microarray data whose expression indicated that they undergo significant changes during *A. viridans* infection. RT-qPCR was used to verify the microarray findings for the following *H. americanus* genes: anti-lipopolysaccharide factor 1, 2 and 4 (ALFHa-1, ALFHa-2, and ALFHa-4), thioredoxin, Acute Phase Serum Amyloid Protein A (SAA), Hexokinase and Trypsin-1b and one gene that was not significant by microarray, Trypsin-1a.

Microarray data and RT-qPCR findings agreed very well with an overall Spearman's Rho of 0.8681. Only hexokinase and trypsin-1a lacked a tight association between the microarray and RT-PCR data as they had Spearman's Rho values less than 0.8 (Table 2.2). When trypsin 1a was removed, because it was not significant after microarray analysis, the Spearman's Rho increased to 0.8871.

The RT-qPCR data also revealed there was differential regulation of all the genes examined at least at some time points (Table 2.2). In most cases, with the exception of trypsin-1a, there was an increase in expression close to, or exceeding, 4-fold as compared to the 6 h control. Of particular interest was the expression of the antimicrobial ALFHA-1, 2 and 4 genes, where ALFHa-1 and ALFHa-2 expression peaked at 4.23, 3.94-fold

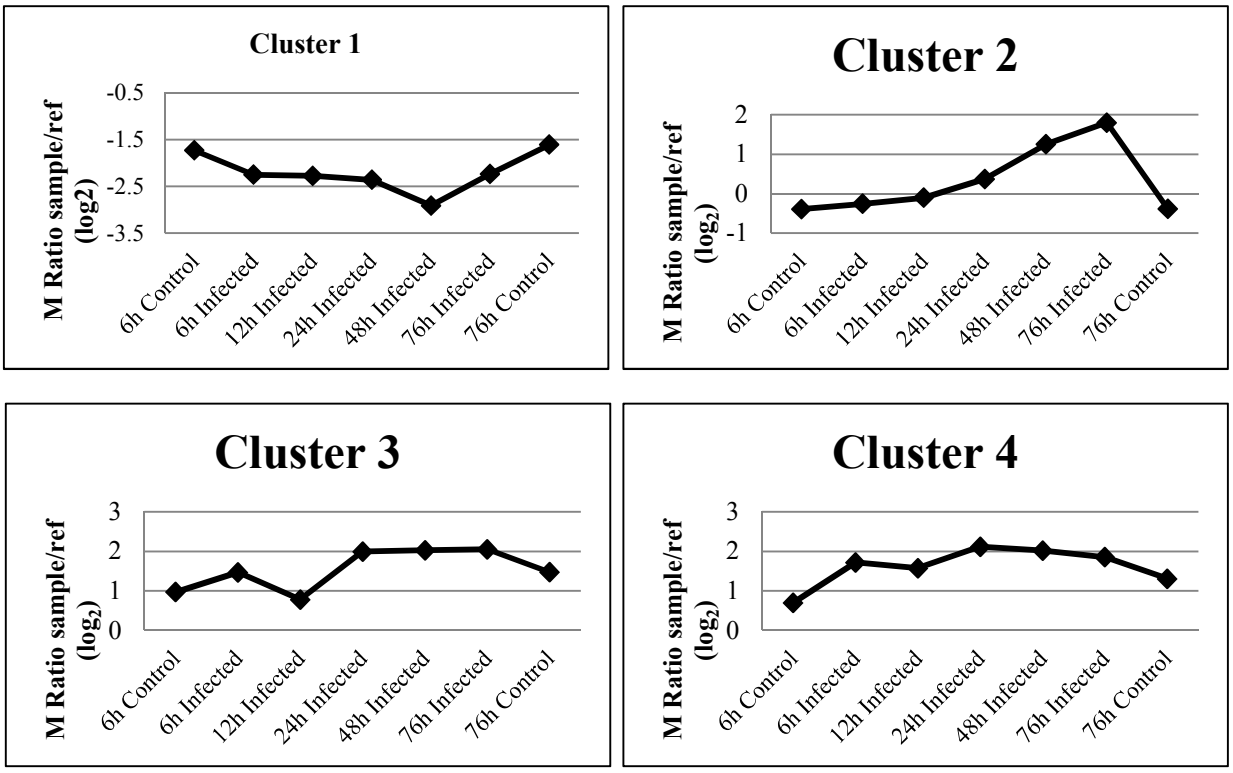

Figure 2.6 K-means cluster analysis of differentially expressed *H. americanus* hepatopancreatic genes as determined by one-way analysis of microarray data at $\alpha = 0.005$.

Table 2.2 RT-qPCR verification of microarray analysis with expression ratios listed as given time-point/6h control except where 76h infected/ 76h control expression ratio is specified

Gene	Accession #	Measurement Technique	Infected					Control	76h Infected/ 76h Control	p-value	Spearman's Rho
			6h	12h	24h	48h	76h	76h			
ALFHa-1	EU625516	RT-qPCR	1.26	1.57	3.19	4.23	3.36	0.27	12.23	3.81E-05	1.000
		Microarray	1.14	1.60	4.03	4.83	4.40	0.58	7.59	0.002	
ALFHa-2	FC556430	RT-qPCR	1.81	2.83	3.48	3.94	3.29	0.25	13.37	3.08E-05	1.000
		Microarray	1.89	2.66	6.42	7.86	6.00	0.64	9.36	<0.000	
ALFHa-4	DV772634	RT-qPCR	1.61	2.09	3.63	12.42	19.84	0.74	26.70	7.48E-08	0.964
		Microarray	0.90	1.37	2.09	3.83	7.15	0.68	10.45	<0.000	
Thioredoxin	FD699182	RT-qPCR	1.44	1.78	1.39	3.68	5.50	0.96	5.71	4.41E-04	0.929
		Microarray	0.90	1.09	1.47	2.81	3.18	0.80	3.97	0.001	
SAA	EH116055	RT-qPCR	6.60	7.41	2.87	6.88	15.31	0.50	30.64	9.12E-03	0.964
		Microarray	1.32	3.04	2.72	3.88	5.44	0.83	6.55	0.004	
Hexokinase	EH401720	RT-qPCR	1.38	0.89	1.03	3.23	6.13	1.59	3.85	0.0118	0.643
		Microarray	1.96	1.01	1.95	2.64	2.59	1.19	2.19	<0.000	
Trypsin 1b	EF095144	RT-qPCR	0.90	2.35	2.25	57.98	69.97	1.29	54.37	1.29E-06	0.714
		Microarray	1.22	1.08	1.68	7.86	9.60	1.38	6.94	0.001	
Trypsin 1a	EV781656	RT-qPCR	1.24	1.26	0.91	0.67	0.54	0.79	0.68	0.0166	0.679
		Microarray	1.04	1.08	1.28	0.87	0.74	0.65	1.13	0.520	

increases at 48 h respectively, while ALFHa-4 peaked at a 19.84-fold increase at 76 h. Acute phase serum amyloid protein A expression peaked at a 15.31-fold increase by 76 h (Table 2.2).

When we compare the 76 h pi infected animals with the 76 h pi controls some additional information begins to stand out. Acute phase serum amyloid protein A, ALFHa-1, ALFHa-2 and ALFHa-4 all have difference in expression increases of 30.64, 12.23, 13.37, and 26.70-fold respectively (Table 2.2). Thioredoxin 1 expression was upregulated 5.71-fold, hexokinase expression was upregulated 3.85-fold and trypsin-1b had the greatest increase in expression of 54.37-fold (Table 2.2).

2.5 Discussion

This is the first study to use a high-throughput genomic technique to analyze the transcriptomic response of the American lobster *H. americanus* during a pathogen challenge. We used a novel oligonucleotide microarray to simultaneously measure the gene expression from 14,592 *H. americanus* genes. The goal was to begin to characterize the breadth and scope of genes involved in the *H. americanus* immunological response to a naturally occurring bacterial pathogen, *A. viridans* var. *homari*.

To date, little is known about what factors are used by *H. americanus* in response to pathogen challenge due to the absence of studies using high-throughput genomic approaches (Hauton, 2012). Genomic analysis of the ESTs that were used to generate our microarray has revealed numerous genes that are widely recognized as classical immune

genes including: 6 ALFHa, 8 crustins, 27 lectins and lectin receptors, 3 masquerade-like proteins, SAA, a peptidoglycan-binding protein, 10 alpha-2-macroglobulins, phenoloxidases, 5 haemocyanins and pseudohaemocyanins, 4 transglutaminases, 8 trypsins and a host of serine proteases and serine protease-inhibitors.

We conducted an *in vivo* infection trial with the Gram-positive bacterial pathogen *A. viridans* var. *homari*. Lobsters were lethally sampled at five time points and tissues were examined histologically and haemolymph was taken for bacterial quantification. Histology and bacterial quantification confirmed that the pathogenesis of disease caused by the *A. viridans* infection progressed as it would in naturally infected animals (Johnson et al., 1981; Battison et al., 2004). Confirmation of the infection model allowed us to monitor the *H. americanus* transcriptome with confidence that the changes we observed were similar to those occurring in naturally infected lobsters.

Lobster hepatopancreatic tissue gene expression was measured for two reasons. Firstly, it contains fixed phagocytes in its terminal arterioles that are effective at removing *A. viridans* from the circulating haemolymph (Johnson et al., 1981), and is a major site for production of humoral immune factors (Factor and Beekman, 1990). One-way ANOVA analysis of microarray data revealed 927 differentially expressed genes at the $\alpha = 0.05$ significance level. Additional Bonferroni correction of the microarray results were performed to significantly reduce the number of false positives that are found due to multiple testing. This correction parameter resulted in 24 genes that were still significantly differentially expressed. However, the Bonferroni correction is a very harsh

correction algorithm and although it significantly reduces the occurrence of false positives, it does so at the expense of true positives, resulting in a large increase in false negatives. For this reason, the one-way ANOVA analysis at the $\alpha = 0.005$ significance level was used in conjunction with biological information to make decisions about which genes are most likely differentially expressed, instead of using a Bonferroni correction.

One-hundred and forty-eight genes were differentially expressed at a significance level of $\alpha = 0.005$. Hierarchical Clustering of the biological samples, both $\alpha = 0.005$ and $\alpha = 0.05$ with Bonferroni correction, was able to highlight several trends in the data (Fig. 2.3 and 2.4). The first is that HC is better at differentiating the control samples from each of the infection sample time points (6 h, 12 h, 24 h, 48 h and 76 h) when a significance level of $\alpha = 0.005$ was used versus at a $\alpha = 0.05$ (Fig. 2.3 and 2.4). Additionally, there was an interesting separation of the 12 h pi samples from the other infected samples with an $\alpha = 0.005$ (Fig. 2.4). Overall, the use of a significance level of $\alpha = 0.005$ and HC with complete linkage was better able to cluster the samples into their respective treatments and time points than the combination of $\alpha = 0.05$ and Bonferroni correction.

Using Blast2GO (B2G) for BLASTx and GO mapping we found that 51.1% of our significant genes share similarity with proteins in GenBank and 46.9% have at least some gene ontology information (Conesa et al., 2005). Although this is a starting point for making functional assignments to *H. americanus* genes, the annotation is severely limited by the lack of well characterized genes for crustaceans, or even arthropods in general. Additionally, a number of genes known to be involved in an immune-response were not

highlighted by the B2G analysis alone. The combination of GO mapping and biological information from primary sources is essential to begin to understand the function of even a small subset of crustacean genes.

The 148 differentially expressed genes can be grouped into four distinct expression patterns (Appendix A Table A.1 and Fig. 2.6). Cluster 2 contains 53 genes which show a progressive increase in expression post-infection during the trial, while the gene expression of the control samples remains stationary. This cluster contains ALFHa-1, ALFHa-4 and ALFHa-6, thioredoxin 1 and SAA genes, all which are widely recognized as being involved in the innate immune response. It is interesting to note that only 41.5% of the genes in cluster 2 have even limited sequence similarity with proteins in GenBank so there are 31 genes in cluster 2 with no previous functional annotation. We can now suggest that they have at least some role in pathogen response in *H. americanus*.

Cluster 4 contains 48 genes, of which 70.8% have some similarity to proteins in GenBank. Four of the genes in cluster 4 have classical immune function and include: ALFHa-2, mannose binding protein, a serine protease inhibitor and haemocyanin subunit 3. Cluster 3 contains 31 genes, of which 35.5% have some similarity to proteins in GenBank and contains a hexokinase and a MRP-like transporter, among other genes. Cluster 1 contains the fewest number of genes at 16, 25% of which have some similarity to proteins in GenBank but none are recognized as classical immune genes.

In addition to the two *H. americanus* ALFs that were characterized by Beale et al., (2008), our microarray measures the expression of four additional ALFHa isoforms that we have named ALFHa-3, ALFHa-4, ALFHa-6 and ALFHa-7. Our microarray findings have found that four ALFHa isoforms, ALFHa-1, ALFHa-2, ALFHa-4 and ALFHa-6, are upregulated in response to *A. viridans* infection. This result was confirmed for ALFHa-1, ALFHa-2 and ALFHa-4 with RT-qPCR. If we compare the RT-qPCR findings of moribund infected animals with their time point controls, we see that ALFHa-4 gene expression increased the most at 26.70-fold, while ALFHa-1 and ALFHa-2 gene expression increased 12.23 and 13.37-fold respectively (Table 2.2). Beale et al., (2008) measured the gene expression of ALFHa-1 and ALFHa-2 in *H. americanus* in response to a Gram-negative bacterial infection with *V. fluvialis*. They found that there was no change in the gene expression of ALFHa-1 and ALFHa-2 in the hepatopancreas at their only sample time point of 24 h; while we have found significant increases in their expression, even at 24 h. Our results highlight the possibility that *H. americanus* mounts a different immune response to Gram-negative and Gram-positive bacterial infections. Perhaps the expression of ALFHa isoforms is even tissue-specific during Gram-negative and Gram-positive bacterial infection, as Beale et al. (2008) found an increase in ALFHa-1, but not ALFHa-2, in gill tissues. Further analysis will be required to determine if ALFHa-1 and ALFHa-2 expression in the gill of our Gram-positive infection model is different than that of the Beale et al. (2008) Gram-negative infection model.

The expression of SAA, as measured by RT-qPCR, demonstrates an interesting, almost biphasic response, where there was an early increase in expression over the first 12 h of

infection, which is typical of an acute phase response, and then a drop in expression to a level similar to the 6 h pi control by 24 h pi. By 48 and 76 h pi, the expression of SAA in infected animals has once again increased so that gene expression is over 30-fold higher in moribund samples than their respective time-point controls. Acute phase serum amyloid protein A is a common marker of inflammation and innate immunological response that is highly conserved in many animals so it is not surprising that this gene would be upregulated during bacterial infection in *H. americanus* (Cray et al., 2009).

Thioredoxin is a protein that is principally concerned with maintaining an intracellular environment in a reduced state. A common response to disease or pathogen infection in crustaceans is the production of free radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) to combat the invading pathogen (Bell and Smith, 1993; Guertler et al., 2010). It is common for *A. viridans* to be phagocytosed by haemocytes or fixed phagocytes where ROS are known to be generated (Moss and Allam, 2006). Our histopathology from the hepatopancreas clearly illustrates that phagocytosis is occurring in *H. americanus* but that they are unable to kill *A. viridans*. The result is proliferation of the bacteria within the phagocytic cells to the point where they rupture the phagocytes and release cellular contents into the circulating haemolymph. This presumably results in a cellular environment with many released or humoral oxidizing factors that can damage host cells. Our RT-qPCR findings indicate that thioredoxin gene expression was increased over 2-fold at 48 h pi and over 5-fold 76 h pi in infected lobster. This increase in thioredoxin gene expression correlated well with the early and nearly complete rupture of hepatic fixed phagocytes at 48 h and 76 h pi, in infected lobsters (Fig. 2.1). It seems

reasonable to suggest that the hepatic expression of thioredoxin at these time points represents an attempt by the host to reduce the damage to host cells in close proximity to the ROS released by ruptured phagocytes, or to control the ROS and RNS generating reactions that are occurring in the lobster haemolymph.

Hexokinase (EC 2.7.1.1) (HK) is an enzyme that catalyzes the first rate-limiting step in glycolysis by phosphorylating glucose. Two HK's have been previously described after isolation from the *H. americanus* hepatopancreas, but their characterization was only at the substrate affinity and protein molecular weight (~50kDa) level (Stetten and Goldsmith, 1981). Without gene or protein level characterization, we are unable to determine which of the two previously described HK gene's expression we are measuring by microarray and RT-qPCR analysis. Hexokinase gene expression initially decreases at 12 h pi and then steadily increases to an over 6-fold increase in expression by 76 h pi. It is well known that *A. viridans* infections are associated with a decrease in dietary carbohydrates, including glucose, in the circulating haemolymph (Stewart, 1984). The increase in HK could be the lobster's attempt to increase glycolysis at a time when haemolymph glucose concentrations are low. Additionally, it has been shown that as *A. viridans* infection progressed in *H. americanus* there is a dramatic loss in hepatic and muscle glycogen reserves (Stewart and Arie, 1973). Glycogen is presumably being converted to glucose and used as an energy source so as glycogen is being rapidly depleted, glycolysis must be upregulated. Therefore HK expression would be increased because it is the first rate-limiting step. A recent study of HK gene expression in *Litopenaeus vannamei* has found that HK expression can be controlled by the hypoxia

inducible factor-1 (HIF-1), where hypoxic conditions increase the HK mRNA level in the gills but not the muscle (Sonanez-Organis et al., 2011). Rittenburg et al., (1979), have studied *A. viridans* infections in *H. americanus* and have found that there is a 50% decrease in the oxygen carrying capacity of haemocyanin which, they postulate, could lead to hypoxia-induced death. It will require further examination to determine if it is an *A. viridans* induced increase in metabolic demand or hypoxia, or both, that cause the increase of HK.

Trypsin-1a and trypsin-1b are both serine proteases, a class of proteins highly associated with crustacean innate immunity (Cerenius and Söderhäll, 2004). Serine proteases mediate the prophenoloxidase cascade which responds to β-1, 3-glycan, lipopolysaccharide and peptidoglycan PAMPs with the production of melanin and toxic metabolites to kill invading microorganisms (Cerenius and Söderhäll, 2004). Microarray analysis determined that the expression of trypsin-1b changes significantly during *A. viridans* infection but the expression of trypsin-1a does not. Trypsin-1a was included for two reasons, the first was to verify that it did not undergo differential expression and the second was to confirm the different expression patterns in these two trypsin isoforms. Trypsin-1b was found to be greatly upregulated at 57.98 and 69.97-fold in the infected lobster at 48 h pi and 76 h pi respectively. This is in stark contrast to trypsin-1a gene expression which remained unchanged. Both of these genes had RT-qPCR expression patterns similar to that observed with the microarray.

We have identified 148 *H. americanus* genes that have significant changes in expression during infection with the Gram-positive bacterium *A. viridans*. This is the first study of *H. americanus* immunity to use microarray technology and we have found excellent correlation between patterns of gene expression in the RT-qPCR and microarray gene expression findings. Although there is discrepancy in the magnitude of gene expression between these two techniques, this finding is common when microarray and RT-qPCR gene expression findings are compared (Morey et al., 2006). This correlation in gene expression patterns suggests that our microarray findings of differential expression for 148 genes are true but the magnitude of gene expression change may be underestimated.

We have monitored the expression of many classical immune genes with our microarray and have found that many are not differentially expressed in the hepatopancreas of our *A. viridans* infected lobsters. These non-significant genes include: 2 ALFHa isoforms, 8 crustins, 27 lectins or lectin receptors, 3 masquerade-like proteins, 1 peptidoglycan recognition protein, 1 β1-3 glucan binding protein, 10 α2-macroglobulins, 1 phenoloxidase, 4 haemocyanins or pseudohaemocyanins, 4 transglutaminases and 7 trypsins. We measured gene expression in the hepatopancreas so the importance of these genes in *H. americanus* immunity would not be found if they are primarily expressed from other tissues. Additionally, these genes may be restricted to the *H. americanus* immune response to pathogens other than *A. viridans*.

This study measured the expression of six ALFHa isoforms and it is interesting to note that only four are differentially expressed. This may represent a pathogen-specific, or at

least a pathogen class-specific response, by *H. americanus* as two of the upregulated genes in our study, ALFHa-1 and ALFHa-2, remained unchanged in the hepatopancreas of lobsters during Gram-negative *V. fluvialis* infection (Beale et al., 2008). Additionally, we have found significant upregulation of the acute phase protein gene SAA, which is highly conserved among animals and has commonly been used as an indicator of an activated immune response in humans and animals (Cray et al., 2009). This study involved the use of only male lobsters so additional study will be required to determine if any gender specific immunological responses exist. It will be interesting to monitor the expression of the SAA gene during different pathogen infections to determine if this indicator of *H. americanus* immune response can be used as a biomarker of lobster health.

2.6 Acknowledgement

We would like to acknowledge the pivotal contribution to this research by the late Dr. David Towle of the Mount Desert Island Biological Laboratory. Dr. Towle's work sequencing the original lobster EST libraries and help generating the first version of our *H. americanus* microarray was immeasurable. David's passion for marine genomics and supportive collegial nature will be sorely missed. The authors also wish to thank the two anonymous reviewers for helping improve the manuscript. Funding for the AVC Lobster Science Centre is provided from multiple sources including: private sector companies, fisher organizations, First Nations and Provincial and Federal Government agencies. Major funding was provided by the Atlantic Innovation Fund (round V) through the Atlantic Canada Opportunities Agency (ACOA) and the PEI Atlantic Shrimp Corporation Inc.

Supplementary data associated with this article can be found in Appendix A.

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3. A transcriptomic analysis of American lobster (*Homarus americanus*) immune response during infection with the bumper car parasite *Anophryoides haemophila*

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K.F. Clark designed and carried out the experiments, analyzed the results and wrote the manuscript. A.R. Acorn provided technical assistance and S.J. Greenwood supervised the work and writing.

3.1 Abstract

Anophryoides haemophila is an important protistan parasite of American lobster, *Homarus americanus*, as it has been found to infect lobsters in the wild as well as causing major losses of lobsters maintained in commercial holding facilities. Expression of over 14,500 *H. americanus* hepatopancreatic genes were monitored during an *A. haemophila* infection challenge in order to elucidate molecular mechanisms involved in the lobster immune response. One hundred and forty-five genes were found to be differentially expressed during infection. For many genes, this study is the first to link their expression to an immune response to a known lobster pathogen. Several of the genes have previously been linked to crustacean or invertebrate immune response including: several anti-lipopolysaccharide factor isoforms (ALFHa), acute phase serum amyloid protein A (SAA), a serine protease inhibitor, a toll-like receptor, several haemocyanin subunits, phagocyte signaling-impaired protein, vitelline membrane outer layer protein-1, trypsin, and a C-type lectin receptor. Microarray results were verified using RT-qPCR and agreement was good between the two methods. The expression of six ALFHa isoforms was monitored via microarray where ALFHa-1, ALFHa-2, ALFHa-4 and ALFHa-6 were

differentially expressed while ALFHa-3 and ALFHa7 were not. RT-qPCR analysis confirmed that ALFHa-1, ALFHA-2 and ALFHa-4 expression increased during infection with a peak at 5-7 weeks for ALFHa-1 and 10 weeks for ALFHa-2 and ALFHa-4. This suggests that different ALFHa isoforms are temporally expressed during *A. haemophila* infection. Importantly, these results provide evidence that different ALFHa isoforms have more significant roles in responding to *A. haemophila* infection. Significant increases in SAA gene expression were also found, corroborating previous findings of increased SAA expression during *A. viridans* infections; highlighting the importance of SAA as a marker of *H. americanus* immune activation and potential indicator of *H. americanus* health.

3.2. Introduction

The adult American lobster (*Homarus americanus*) has relatively few known lethal pathogens (Cawthorn, 2011). *Anophryoides haemophila* is a naturally occurring scuticociliate known to infect lobster in the wild, however nothing is known about the natural mortality that this causes (Cawthorn, 1997, Lavallée et al., 2001). This protistan parasite has been widely recognized as a significant concern in cold water storage facilities (<5 ^{o}C) where lethal infections are known to cause substantial losses (Loughlin et al., 1993; Cawthorn, 1997; Greenwood et al., 2005). A previous study has looked at the transcriptome of *A. haemophila* for clues as to how it mediates entry into *H. americanus* but no definitive mechanism was found (Acorn et al., 2011).

Very little is known about how the *H. americanus* humoral immune system is able to mediate its protection to the abundant opportunistic pathogens present in the marine

environment. We previously demonstrated the importance of 148 genes in the *H. americanus* response to the Gram-positive pathogen *Aerococcus viridans* var. *homari* (Chapter 2) This was the first time that many of these genes were implicated in *H. americanus* immunity and many new isoforms of previously known immune genes were highlighted including: six anti-lipopolysaccharide factor (ALFHa) isoforms, acute phase serum amyloid protein A (SAA), thioredoxin, and trypsin (Chapter 2). Other studies have found a small number of *H. americanus* immune factors including two antimicrobial peptides (Battison et al., 2008), four plasma lectins (Battison and Summerfield, 2009), ALFHa-1 and ALFHa-2 (Beale et al., 2008) and a crustin (Christie et al., 2007). Historically, haemolymph based agglutinin factors have also been studied but the absence of protein or nucleotide sequence information make it difficult to identify the particular genes or proteins involved (Cornick and Stewart, 1973; Hall and Rowlands, 1974a; 1974b; Hartman et al., 1978; Abel et al., 1984).

Hauton (2012) recently noted that the lack of annotated expressed sequence tag and genomic information related to crustaceans has been an impediment to crustacean immune factor discovery. While our previous study discovered genes involved in a humoral immune response in lobster to a Gram-positive bacteria, it is still unknown whether the lobster immune system reacts differently when infected with other types of pathogens.

The goal of this study was to discover novel humoral immune factors used by *H. americanus* to combat parasitic infection. Hepatopancreatic tissue was monitored for

changes in gene expression during *A. haemophila* infection because it is a major source of humoral immune factors (Factor and Beekman, 1990). Although *A. haemophila* does not target the hepatopancreas, and *H. americanus* mounts a significant cellular immune response, (Athanassopoulou et al., 2004, Greenwood et al., 2005), it follows that humoral factors are likely playing a role in either initially inhibiting the growth and spread of the parasite or binding to *A. haemophila* to encourage haemocyte aggregation and encapsulation.

Previously, we investigated the *H. americanus* immune response to a warm water (15 ^{o}C) Gram-positive bacteria (Chapter 2), while this current study investigated immune responses to a cold water (2 ^{o}C) ciliated protist. Gene expression of hepatopancreatic tissue was monitored with a *H. americanus* microarray during *A. haemophila* infection to identify molecular mechanisms responsible for lobster humoral immune response to parasitic infection.

3.3. Materials and Methods

3.3.1 Animal Handling

Adult male American lobsters, *H. americanus*, (n = 48, 548 g ± 11.9 g) were purchased from a local seafood supplier (Bedford, Nova Scotia) and were assessed for physical and clinical indicators of health to ensure only healthy, pathogen-free lobsters were used (Acorn et al., 2011). Lobsters were housed individually in recirculating artificial seawater (ASW) (Instant Ocean, 30 ppt) system equipped with UV filtration (Aquabiotech, Coaticook, QC, Canada) in the Aquatic Animal Facility of the Atlantic Veterinary

College. Lobsters were handled in accordance with the University of Prince Edward Island Animal Care Committee approved animal care protocol #09-052. Lobsters were acclimated to 2 °C by decreasing the water temperature from 6 °C by 1 °C per day to mimic the water temperature where natural infections of *A. haemophila* usually occur. Lobsters were held for two weeks prior to the beginning of the experiment, which included the acclimation period. Lobster were not feed during the trial.

3.3.2 *A. haemophila*

A virulent strain of *A. haemophila* (Ah-6, Nova Scotia 2004) was initially isolated from infected lobsters (Greenwood et al., 2005) and has been maintained in modified marine agar medium (ATCC 1651, Messick and Small, 1996; Acorn et al., 2011) at 4 ^{o}C. *A. haemophila* were centrifuged at 500*g* for 5 min at 4 °C and washed twice with sterile (0.20 µm filtered) 3% ASW. The *A. haemophila* suspension was checked to ensure parasite viability and diluted to 5×10^5/mL by counting with a haemocytometer and diluting with additional sterile 3% NaCl.

3.3.3 *A. haemophila* Infection Challenge

Lobsters were randomly assigned to receive either 200 µL injections of 5×10^5/mL *A. haemophila* (n = 38) or sterile 3% NaCl (n = 10). *A. haemophila* challenged lobsters were randomly selected (n = 4) for lethal sampling at 24 h, 48 h, 1 week, 3 weeks, 5 weeks, 8 weeks and 10 weeks when the remaining infected lobsters became moribund. Several infected lobsters became moribund and had to be sampled early and were included in the time treatment in which they were euthanized (n = 11). Lobsters that received the sterile

3% NaCl injections were euthanized at 24 h (n = 3), 8 weeks (n = 3) and 10 weeks (n = 4).

3.3.4 *H. americanus* Sampling

Weekly haemolymph sampling was conducted to determine the concentration of circulating haemocytes and to detect the presence of circulating *A. haemophila* as described in Battison et al. (2003). *H. americanus* were lethally sampled by severing the ventral nerve cord as per Chapter 2. Haemolymph was obtained to determine the presence of *A. haemophila* in the circulating haemolymph, hepatopancreatic tissue was taken for RNA analysis while 10 tissues were collected for histologic analysis including: hepatopancreas, heart, gill, antennal gland, testis, stomach, intestine, ventral nerve cord, tail muscle and claw muscle.

Lobster hepatopancreatic tissue was preserved for microarray and RT-qPCR analysis by immediate homogenization in RNA preservation reagent (1.4 M guanidine isothiocyanate, 38% phenol (pH 4), 5% glycerol and 0.1 M sodium acetate) at a ratio of less than 100 mg tissue/mL RNA preservation reagent. Homogenized tissue was then flash frozen in liquid nitrogen and stored at -80 °C until used.

RNA was isolated as previously described (Chapter 2). Briefly, 200 µL of chloroform per mL of RNA preservation reagent was added to thawed tissue homogenate, shaken for 10 s, incubated at room temperature and then centrifuged at 12,000*g* for 15 min at 4 °C. Equal parts of the resulting supernatant was mixed with 70% ethanol and added to an

RNeasy spin column (Qiagen, Toronto, ON, Canada). RNA was isolated following the manufacturer's instructions including the optional DNase I (Qiagen) treatment. RNA was quantified using a NanoDrop-1000 (Thermo Fisher, Ottawa, ON, Canada) and the quality of the RNA was determined using an Agilent Bioanalyzer microcapillary electrophoresis unit (Agilent, Mississauga, ON, Canada). RNA samples were stored at -80 °C until used.

Tissues for histology were preserved in 10% formalin in 0.2 µm filtered ASW then dehydrated and infused with paraffin wax using an infiltration processor and standard protocols. A rotary microtome was used to cut serial sections (3-5 µm) which were mounted on glass slides and stained with haematoxylin and eosin (H&E). Stained sections were evaluated using a light microscope (Zeiss Axioplan 2).

3.3.5 *H. americanus* Microarray Design and Construction

The *H. americanus* microarray used in this project has previously been described in detail (Chapter 2). Briefly the array contains 14,592 50-mer DNA probes designed from EST sequences and *H. americanus* sequences in GenBank. Many control spots are also present on the array including: 210 Alien DNA controls, 78 GFP controls, 80 GFP landmarks and 416 buffer controls, for a total of 15,376 spots.

3.3.6 cDNA Labeling and Microarray Hybridization

Alexa Fluor® 555 labeled single strand cDNA was generated from 20 µg high quality sample RNA using 20 µg of RNA using a SuperScript™ Plus Indirect cDNA Labeling

System (Life Technologies Inc., Burlington, ON, Canada). Reference aRNA was generated from 5 µg of pooled lobster hepatopancreatic RNA using an Amino Allyl MessageAmp™ II aRNA Amplification kit (Life Technologies Inc.). Reference aRNA was Alexa Fluor® 647 labeled using 12 µg of aRNA and a Superscript™ Plus Indirect cDNA Labeling System (Life Technologies Inc.). Labeled aRNA and cDNA were quantified using a NanoDrop ND-1000 and 140 ng of cDNA and 100 pmol of labeled aRNA were used for microarray hybridization. Labeled aRNA was fragmented using 1 µL 10× Fragmentation solution and incubation for 10 min at 70°C followed by 1 µL of Stop solution (Life Technologies Inc.). Hybridizations were performed using A2 chambers on a Tecan 400 HS Pro hybridization system (Tecan, NC, USA) as previously described (Chapter 2).

3.3.7 Microarray Data Analysis

Microarrays were imaged using a GenePix 4000B scanner (Molecular Devices, PA, USA), features were extracted using SpotReader v 1.3.1 (Niles Scientific, CA, USA) and resulting data was analyzed using TM4/MeV v4.8.1 (Saeed et al., 2003). LOWESS was used to normalize two color gene expression arrays and flagged using MIDAS (Microarray Data Analysis Software) v2.2.2 (Quackenbush, 2002). Log2 expression ratios of sample to reference gene intensity was generated to compare arrays with one way ANOVA analysis (MeV) at $\alpha = 0.05$ with and without Bonferroni correction and at $\alpha = 0.005$ without Bonferroni correction. Figure of Merit (FOM), K-means clustering and Hierarchical Clustering (HC) were used to determine similarity between the expression profiles of significantly differentially expressed genes (Soukas et al., 2000; Yeung et al.,

2001). All raw and normalized microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GSE43587 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43587).

3.3.8 Verification of Microarray Results

Microarray findings were verified using reverse transcriptase quantitative polymerase chain reactions (RT-qPCR) to measure the mRNA expression of 10 selected genes of interest by RT-qPCR: *H. americanus* antilipopolysaccharide factors (ALFHa-1, ALFHa-2, ALFHa-4), acute phase serum amyloid protein (SAA), C-type lectin receptor, pseudohaemocyanin-2 precursor, phosphoenolpyruvate carboxykinase 1 (PEPCK-1), trypsin-1a, trypsin-1b and thioredoxin. Normalization genes were validated using geNorm^{PLUS} in the qbase^{PLUS} qPCR analysis software suite v2.3 (Biogazelle, Belgium). A total of 2 genes (Coagulation factor VIII-associated protein (FD699377) and Lava lamp protein (CN853553) were determined to be the minimal number of normalization candidates needed based on them having an average geNorm^{PLUS} M value of 0.359 and an average geNorm^{PLUS} V value of 0.126.

RT-qPCR was performed as previously described (Chapter 2). Briefly, cDNA was generated from 1 µg of high quality sample RNA using the Superscript III First Strand Synthesis kit (Life Technologies Inc.), with oligo d(T) primers as per the manufacturer's instructions. qPCR reactions were performed using 2 µL of cDNA in a total reaction volume of 15 µL using a Chromo4 Real-Time PCR system (BioRad, Hercules, CA).

qPCR contained 1x Express SYBR GreenER with ROX (Life Technologies Inc.) and 200 nM of each primer (Appendix B Table B.1).

Two-step and three-step qPCR reactions were used in order to meet our reaction efficiency and reproducibility quality standards. Two-step qPCR protocols were as follows: 50 °C for 2 min 95 °C for 2 min followed by 39 cycles of 95 °C for 7 s then 20 s at the gene-specific annealing temperature (Appendix B Table B.1) followed by a plate read. Three-step qPCR protocols were the same as two-step reactions with the addition of 72 °C for 20 s after each annealing step. Product specificity was assessed with melt curves between 65-90 °C in 1°C increments every 2 s to ensure single peaks. Amplicons were analyzed on 2% agarose gels to confirm proper size. All samples were run in triplicate for qPCR analysis and every run contained negative RT and negative template controls to confirm the absence of contaminating genomic DNA.

3.4 Results

3.4.1 Confirmation of *A. haemophila* Infection

Multiple clinical and histological methods were used to confirm *A. haemophila* infection in *H. americanus* in our experimental, and absence in our control, groups. Weekly total haemocyte counts (THC) demonstrate that there was a decrease in circulating haemocytes in the infected animals that began at week 5 and was statistically significant by week 10 ($p < 0.05$) when the remaining infected lobsters became moribund (Fig. 3.1). Live *A. haemophila* were detected in infected animals 7 days after injection and for the remainder of the trial. *A. haemophila* was not detected in the haemolymph of any control animals or

the 24 or 48 h infected animals. Histological examination of heart, gill, hepatopancreas, stomach, testis, antennal gland, intestine, and claw and tail muscle found *A. haemophila* sporadically in tissues of infected animals within the first five weeks of the trial, and in all infected animals after 5 weeks. *A. haemophila* were detected earliest, and most commonly, in gill tissues, but were also found in the heart and connective tissues of the testis in higher numbers than all other tissues, throughout the infection (Fig. 3.2).

Microscopic examination revealed a strong cellular immune response in *H. americanus* where circulating haemocytes were recruited to areas where *A. haemophila* were present and initiated encapsulation cascades around the parasites (Figs. 3.2 and 3.3). Haemocyte aggregations were absent in control lobster tissues but were very common in the first five weeks of the trial in *A. haemophila* infected lobsters. After five weeks of infection it became increasingly common to find *A. haemophila* without haemocytes in close proximity and there was an inverse correlation between the number of visible encapsulation events and free *A. haemophila* (Figs. 3.2 and 3.3). This finding coincided temporally with haemocytopenia.

3.4.2 Microarray Analysis of Differential Gene Expression

Hepatopancreatic tissue was monitored for transcriptomic changes in gene expression during *A. haemophila* infection. One-way ANOVA, in conjunction with sample HC was used to determine the statistical stringency that yields the most biologically meaningful differential expression information. One-way ANOVA with an $\alpha = 0.005$ found 145 genes whose gene expression was significantly altered over the course of our experiment

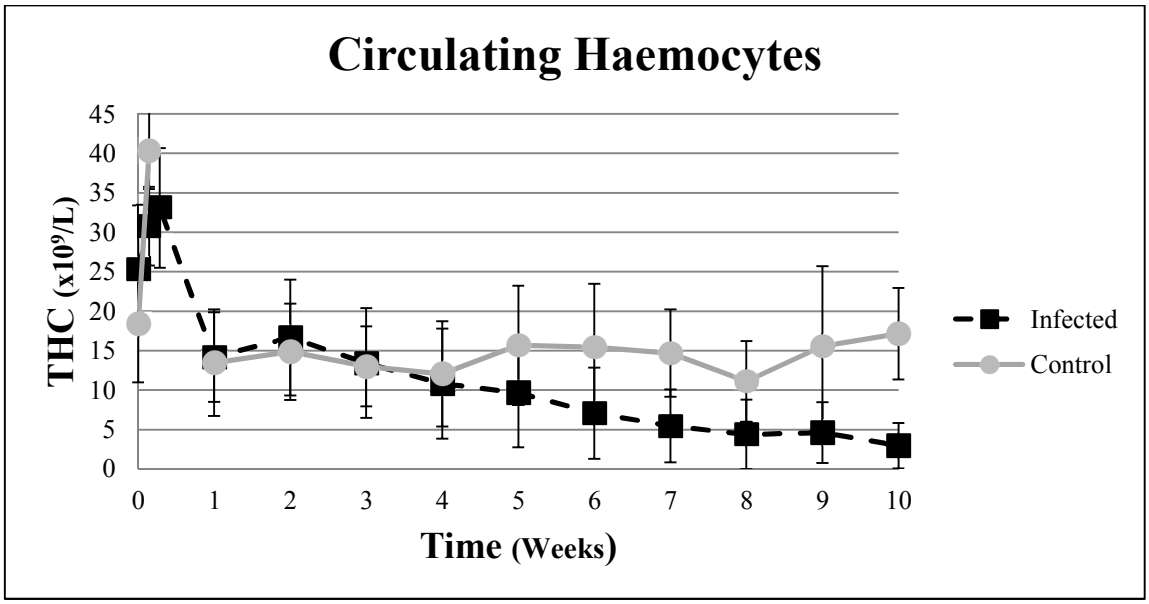

Figure 3.1 Circulating haemocytes in *Homarus americanus* during the *Anophryoides haemophila* challenge. (THC = total haemocyte count, n = 4)

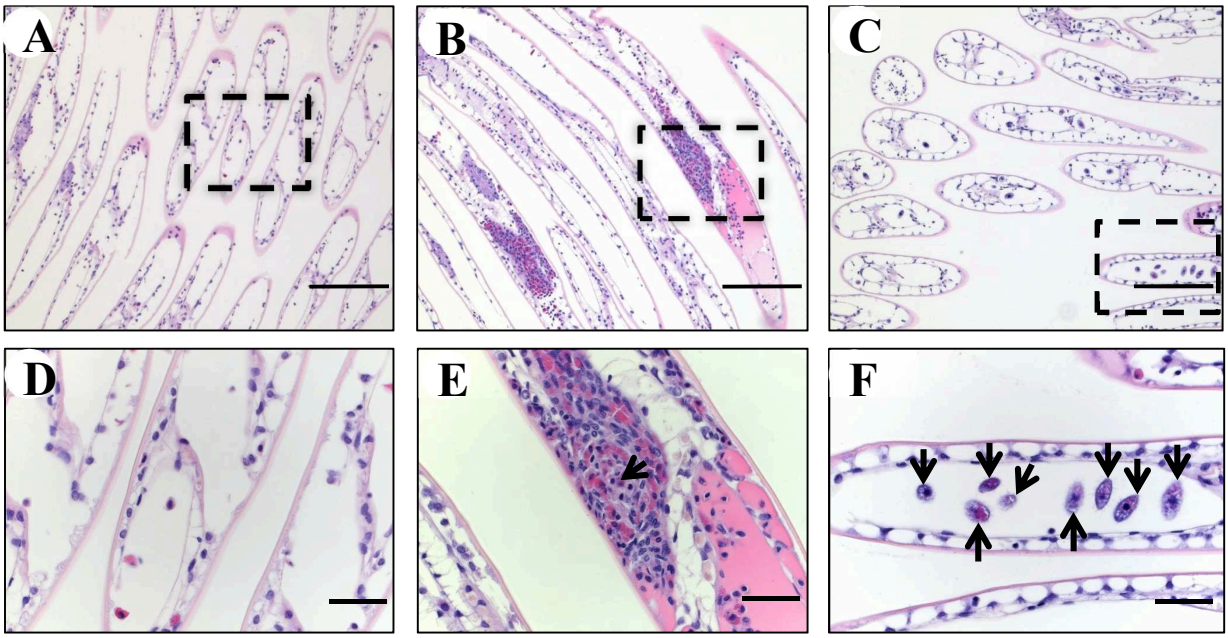

Figure 3.2 Histopathology of *Homarus americanus* gill tissue during infection with *Anophryoides haemophila*. (A) and (D) indicate typical findings in control animals at 24h, (B) and (E) indicate typical findings at 1-5 weeks and (C) and (F) indicate typical findings in moribund animals. Broken line boxes in (A), (B) and (C) area of magnified image in (D), (E) and (F) respectively. Arrows denote *Anophryoides haemophila*. Black bar represents 50 µm.

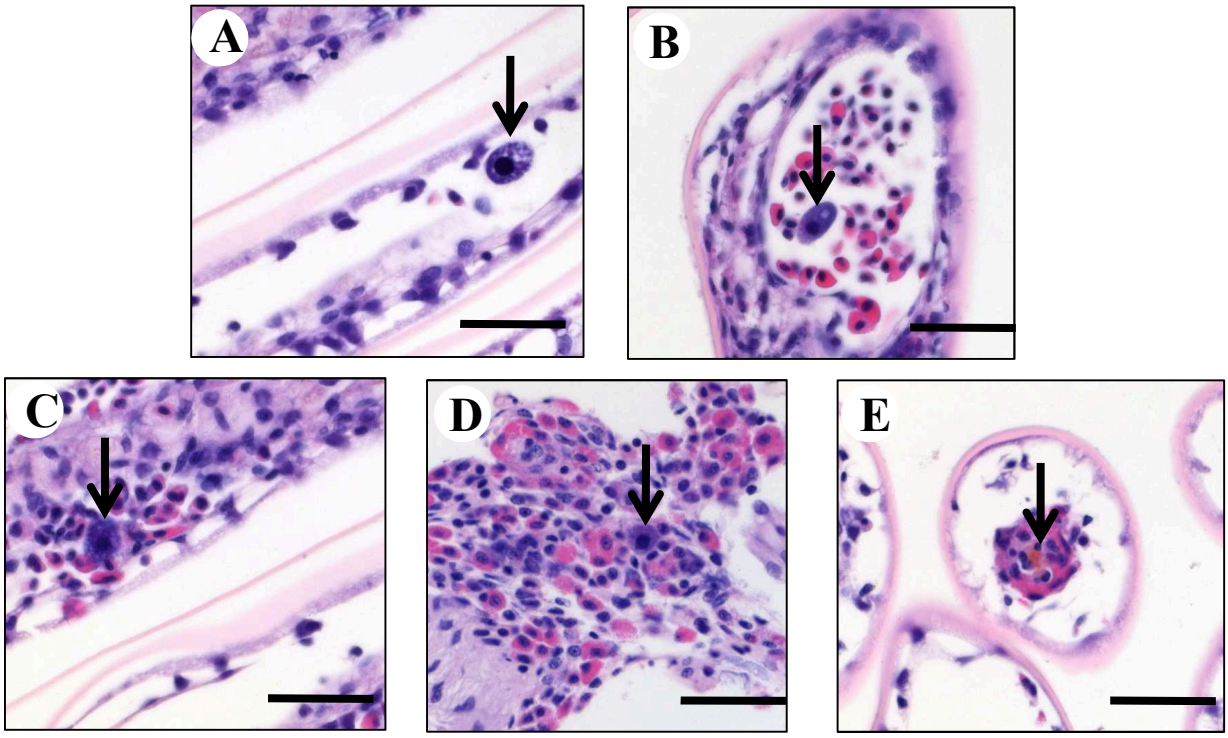

Figure 3.3 Histopathology of *Homarus americanus* cellular immune response in gill tissue after infection with *Anophryoides haemophila*. All plates depict the progression of circulating haemocyte mediated encapsulation of *A. haemophila* from early recognition of pathogen (A) to pathogen destruction by haemocyte mediated generation of toxic metabolites (E). Bold arrows indicate *A. haemophila*. Black bar represents 50 µm.

(Appendix B Table B.2). When we decreased α to 0.05 we found 928 differentially expressed genes but a high probability of false-positives due to multiple testing errors. When the statistical stringency was increased with Bonferroni Correction of the $\alpha = 0.05$ results, we found 38 differentially expressed genes but an unacceptable number of false-negatives.

The sample HC generated from the 38 Bonferroni corrected genes cluster loosely into two very early time points encompassing controls and infected animals from 24 h to 3-4 weeks, a late infected time point cluster and cluster with 8 week controls (Appendix B Fig. B.1). The sample HC generated with the 145 genes differently expressed at $\alpha = 0.005$ cluster into 4 time points with one consisting of early in the infection, one late in the infection and two smaller clusters containing the controls and predominantly an infected treatment sample from most time-points (Appendix B Fig. B.1). Sample HC of differentially expressed genes at the various levels of statistical stringency illustrates that one-way ANOVA at $\alpha = 0.005$ gives the most biologically relevant information as it correlates most with treatment (Appendix B Fig. B.2).

The 145 significantly differentially expressed genes were clustered into groups based on the similarity of their gene expression profiles to gain additional insight into gene function. Figure of Merit analysis determined that there were 7 relevant gene expression profiles and K-means clustering was used to generate these 7 clusters (Fig. 3.4). Additional functional information can be garnered by using HC to cluster the samples of the differentially expressed genes as it is highly probable that clustered genes have

expression that is linked, or co-regulated, and therefore have similar function or biological importance (Eisen at al., 1998) (Figs. 3.5 and 3.6).

Cluster 3 consists of 20 genes including: 3 haemocyanin and 2 pseudohaemocyanin genes, C-type lectin receptor, vitelline outer layer protein-1 like protein (VOM-I) and a Heat shock protein (Hsp) 70. Samples cluster into an early time point less that 5 weeks, and a late time point longer than 5 weeks irrespective of infection status (Fig. 3.5). Cluster 6 contains 30 genes with none of them known to play a direct role in mediating immunity. Sample HC largely separates the infected from the control samples but the two clusters containing infected samples are not separated temporally (Fig. 3.6). Cluster 7 contains 18 genes including several well-known immune genes: ALFHa-6, ALFHa-4, SAA, and a Toll-like receptor. Sample HC of cluster 7 separates into three groups consisting of infected samples from 5-10 weeks, infected samples from 24 h to 4 weeks and the 10 week control and finally a cluster containing the 24 h and 8 week control (Fig. 3.6).

3.4.3 Verification of Differential Gene Expression Using RT-qPCR

RT-qPCR was used to confirm that genes found to be differentially expressed via our *H. americanus* microarray were truly differentially expressed. Ten genes were used for microarray verification including: ALFHa-1, ALFHa-2, ALFHa-4, SAA, C-type lectin receptor, pseudohaemocyanin-2 precursor, PEPCK-1, trypsin-1a, trypsin-1b and thioredoxin (Table 3.1).

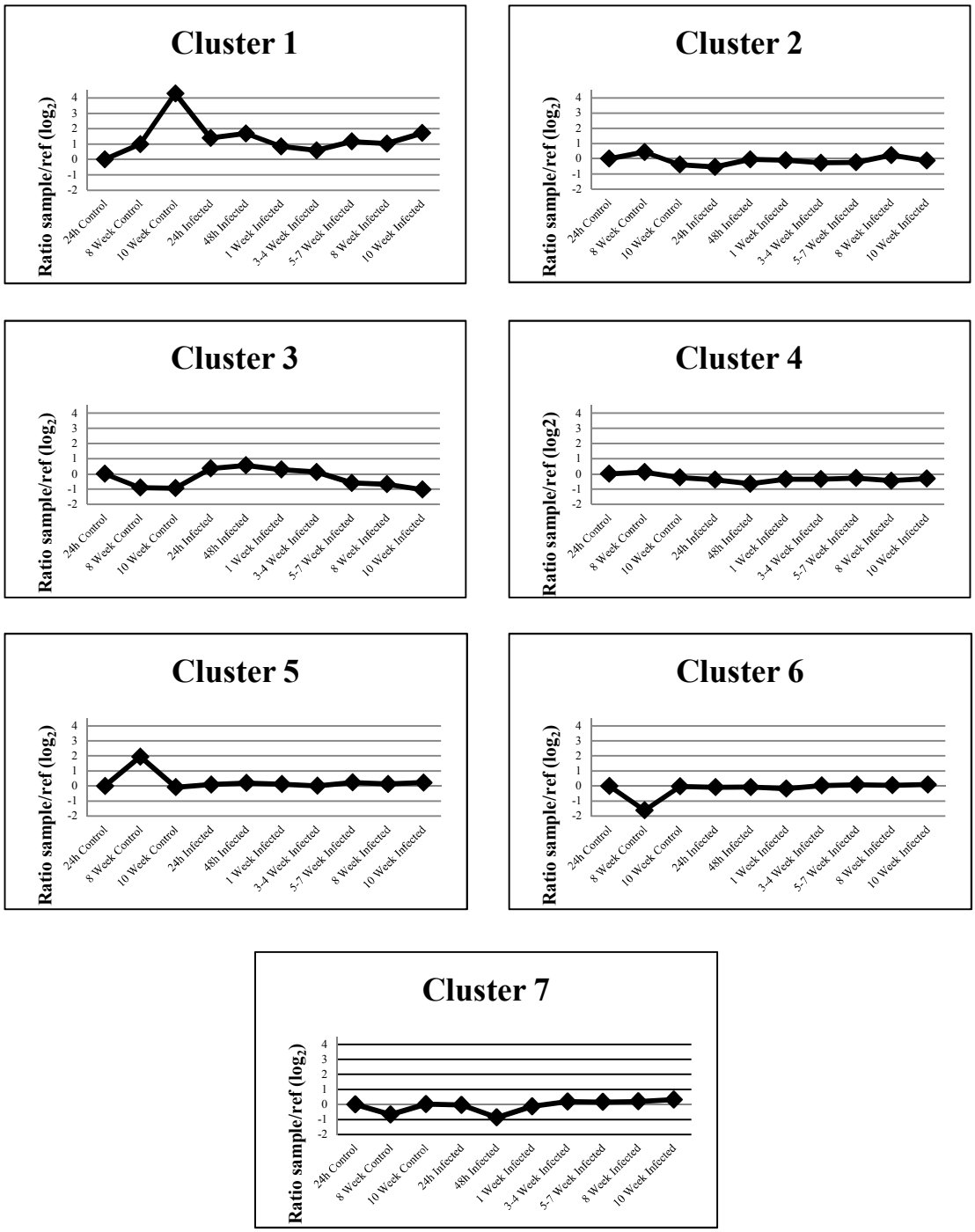

Figure 3.4 Gene clusters of the 145 differentially expressed genes at $\alpha = 0.005$ as generated by K-means. Gene clusters are generated based on the similarity of their gene expression profiles during the different treatments and treatment times. The magnitude of the gene expression changes that occur within each cluster can be easily compared based on their position on the vertical axis.

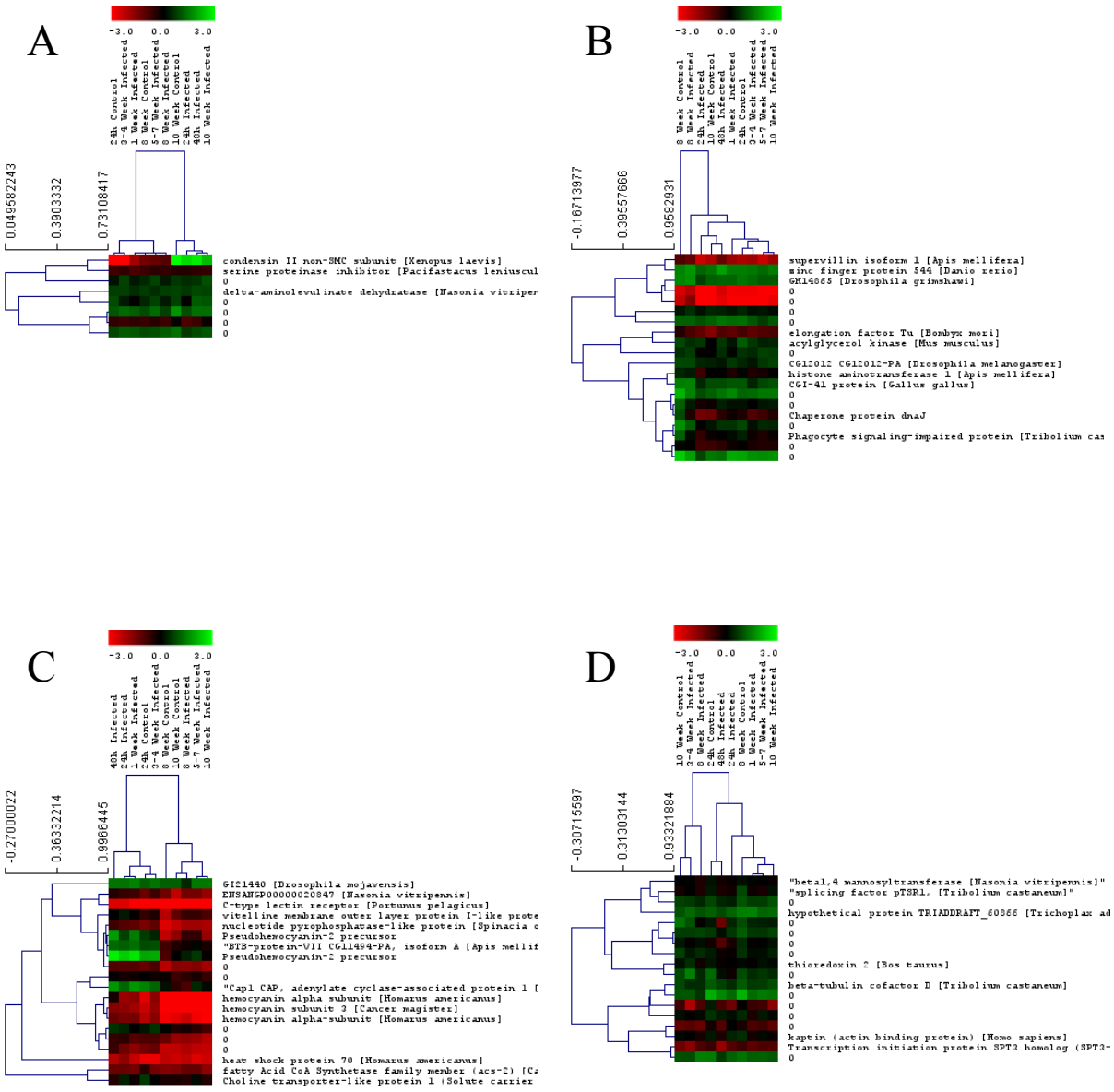

Figure 3.5 Sample Hierarchical Clustering of the K-means gene clusters generated from the 145 differentially expressed genes at $\alpha = 0.005$. (A) cluster 1, (B) cluster 2, (C) cluster 3, (D) cluster 4. Gene expression heat maps of the ratio of gene expression where a gradient of red to green represents a three-fold or greater decrease in gene expression to a three-fold or greater increase in gene expression. Columns represent the average gene expression of a given treatment at each gene displayed horizontally.

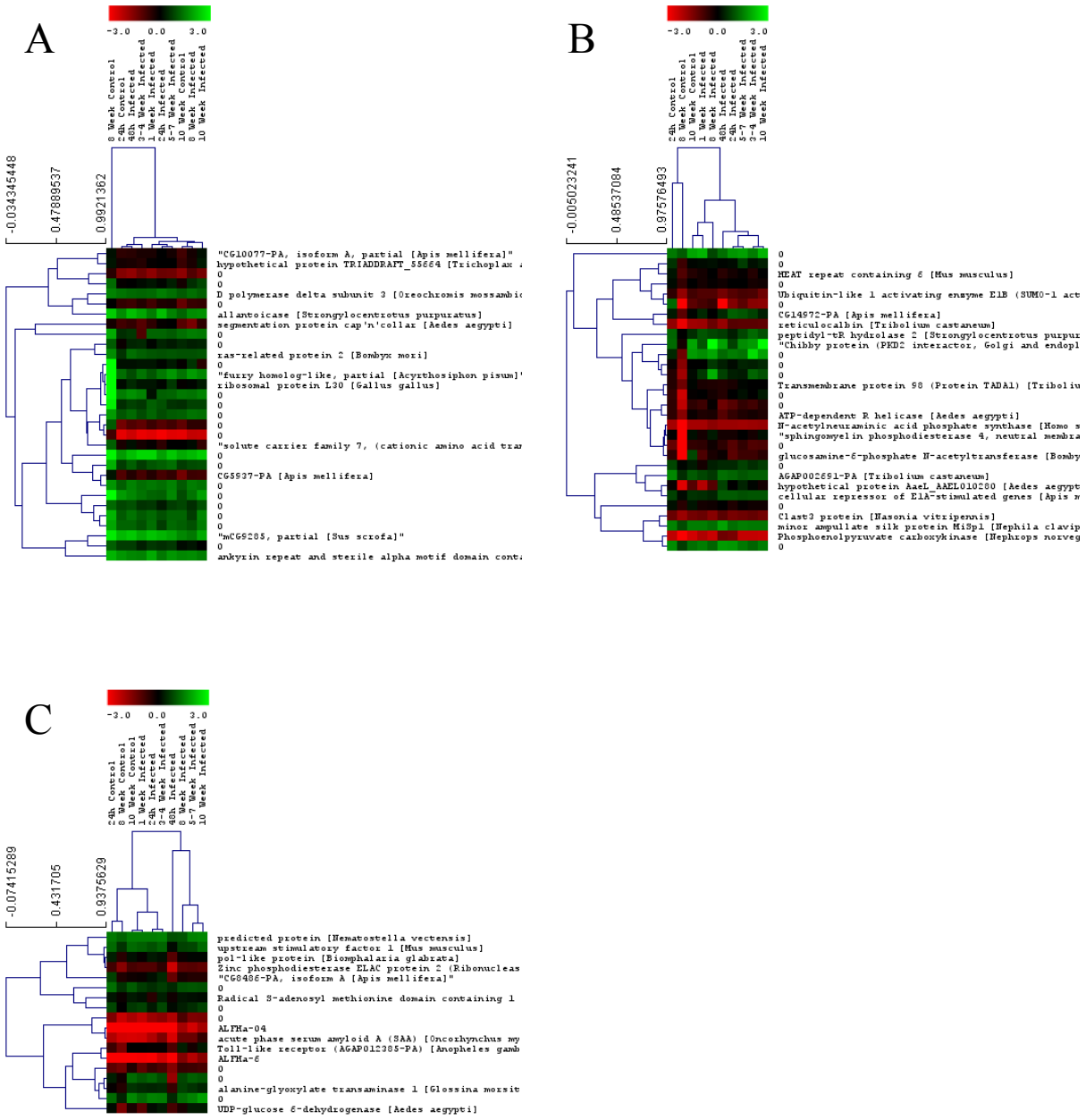

Figure 3.6 Sample Hierarchical Clustering of the K-means gene clusters generated from the 145 differentially expressed genes at $\alpha = 0.005$. (A) cluster 5, (B) cluster 6, (C) cluster 7. Gene expression heat maps of the ratio of gene expression where a gradient of red to green represents a three-fold or greater decrease in gene expression to a three-fold or greater increase in gene expression. Columns represent the average gene expression of a given treatment at each gene displayed horizontally.

The microarray and RT-qPCR data agree quite well with an average Spearman's Rho of 0.862 and 0.830 for genes significant at $\alpha = 0.005$ and $\alpha = 0.05$ respectively. There is only poor agreement between microarray and RT-qPCR analysis of ALFHa-1 gene expression, with a Spearman's Rho of 0.539, even though both methods find clear differential expression with p-values of 0.027 and 6.56×10^{-9}. When ALFHa-1 is removed from the microarray and RT-qPCR comparison, the average Spearman's Rho increases to 0.872. Thioredoxin expression is not significant using either microarray or RT-qPCR, nor do the findings of the two methods correlate with each other with a Spearman's Rho of 0.212.

RT-qPCR analysis revealed that all of the genes that were significant at $\alpha = 0.005$, and those that have previously been implicated as having a role in *H. americanus* immunity, were significantly differentially expressed at least at some time points during the trial. All of the ALFHa isoforms had their lowest expression at 48 h, while ALFHa-1 expression peaked at 5-7 weeks and ALFHa-2 and ALFHa-4 expression peaked at 10 weeks. SAA expression steadily rose from the early 24 and 48 h time points to a peak expression of 22.14-fold that of the 24 h control at 10 weeks. ALFHa-1, ALFHa-4 and SAA, but not ALFHa-2, peak expression is statistically significant at $\alpha = 0.05$ when compared to the 24 h control. Comparison of ALFHa-1, ALFHa-2, ALFHa-4 and
SAA expression for infected vs. control samples at 10 weeks is significant at $\alpha = 0.05$ with values of 6.73-, 35.56-, 8.40- and 54.94-fold respectively.

C-type lectin receptor expression was significantly upregulated in the infected compared to the control animals at 24 h with an increase of 5.06-fold. The expression of C-type

Table 3.1 RT-qPCR verification of microarray analysis with expression ratios listed as given time-point/24h control except where specified.

Gene	Accession #	Measurement Technique	Infected							Control		10 wk Infected/ 10 wk Control	p-value	Spearman's Rho
			24h	48h	1 wk	3-4 wk	5-7 wk	8 wk	10 wk	8 wk	10 wk			
ALFHa-1	EU625516	RT-qPCR	1.57	1.39	2.66	2.81	4.79	4.28	3.18	0.44	0.47	6.73	6.56E-09	0.539
		Microarray	0.54	0.42	0.70	0.78	0.98	0.95	0.83	0.55	0.45	1.85	0.027	
ALFHa-2	FC556430	RT-qPCR	2.16	1.36	4.20	5.06	10.15	10.91	11.52	0.63	0.32	35.56	6.60E-07	0.842
		Microarray	0.93	0.63	1.34	1.45	1.98	1.85	2.58	1.21	0.93	2.78	0.011	
ALFHa-4	DV772634	RT-qPCR	0.76	0.67	3.03	2.53	3.40	4.62	5.11	0.35	0.61	8.40	8.21E-10	0.709
		Microarray	0.55	0.46	0.63	0.79	1.11	1.26	1.54	0.81	0.60	2.58	0.005	
SAA	EH116055	RT-qPCR	0.80	0.74	1.68	6.47	13.94	18.99	22.14	1.00	0.40	54.94	2.08E-09	0.770
		Microarray	0.70	0.47	0.54	1.02	1.19	0.95	1.80	0.76	0.63	2.88	0	
C-type lectin receptor	CN852574	RT-qPCR	5.06	2.20	2.08	0.90	0.67	0.34	0.31	0.71	0.42	0.74	4.42E-07	0.915
		Microarray	2.41	2.24	1.94	1.09	0.80	0.52	0.70	1.13	0.70	1.00	0	
Pseudohaemocyanin-2 precursor	CN952339	RT-qPCR	0.81	1.59	0.87	0.64	0.23	0.16	0.10	0.07	0.10	0.97	9.45E-05	0.952
		Microarray	0.96	1.15	1.21	0.70	0.28	0.17	0.15	0.14	0.21	0.73	0.001	
Phosphoenolpyruvate carboxykinase 1	DV773064	RT-qPCR	1.69	1.55	1.09	0.50	0.64	0.46	0.48	0.36	0.64	0.75	2.74E-04	0.964
		Microarray	1.23	1.24	0.80	0.65	0.64	0.48	0.60	0.27	0.74	0.81	0	
Trypsin 1a	EV781656	RT-qPCR	2.24	1.63	1.34	0.76	0.77	0.85	0.83	0.63	0.72	1.15	6.42E-03	0.770
		Microarray	2.34	3.19	1.13	1.23	0.93	0.90	1.00	0.63	0.77	1.30	0.037	
Trypsin 1b	EF095144	RT-qPCR	1.14	0.40	0.59	0.65	1.68	2.39	1.08	0.19	0.10	10.57	6.42E-03	0.952
		Microarray	0.83	0.48	0.59	0.73	1.32	2.09	1.06	0.22	0.39	2.74	0.003	
Thioredoxin	FD699182	RT-qPCR	1.62	1.20	1.41	1.45	1.63	1.60	1.69	1.18	1.15	1.47	9.05E-02	0.212
		Microarray	0.61	0.61	0.42	0.65	0.79	0.88	0.84	0.61	0.58	1.44	0.783	

lectin receptor decreases throughout the trial with the expression in the infected animals at 24 h higher than at any time-point of 3 weeks or later. Expression in infected animals at 48 h, and 1 week, is higher than at 8 and 10 weeks.

Pseudohaemocyanin-2 precursor expression is never different than its corresponding time-point controls. However, there is a significant decrease in expression in the infected 10 week animals, compared to 24 h animals, of 8.32-fold. There is also a significant difference between control animals at 24 h and 8 weeks at 14.08-fold, but there is no significance at 10 weeks, even with a decrease of 10.00-fold.

PEPCK-1 expression is upregulated at 24 h when compared to the 3-4 week and subsequent infected time points and the 8 week control with expression differences ranging from 2.64- to 4.69-fold. PEPCK-1 48 h expression is significant when compared to the 8 week controls and the 3 week and all subsequent infected time points, with the exception of 5-7 weeks, with expression differences of 3.1- to 4.3-fold.

Trypsin-1a has a slight increase in expression at 24 h that is statistically significant from the infected and control samples from 3 weeks to the end of the trial, with the exception of 8 week infected, at 2.70- to 3.56-fold. Trypsin-1b expression is significantly higher for the 5-7 week, 8 week and 10 week infected over the 10 week control at 16.80-, 23.90- and 10.80–fold respectively. Although the 8 week infected is 10.86-fold higher than the 8 week control, it is not significant at $\alpha = 0.05$.

Thioredoxin expression was not found to be significantly different using microarray analysis and this was confirmed using RT-qPCR.

3.5 Discussion

This is the first study to characterize the differential gene expression response of *H. americanus* to a protistan parasite, *A. haemophila*, using a high-throughput transcriptomic approach. Very little is known about the *H. americanus* immune system but a transcriptomic approach monitoring the immune response during an established infection by a natural pathogen was viewed as the best way to significantly expand our understanding of the lobster immune system. The *A. haemophila* challenge in this study followed normal disease progression and pathology where the ciliates were initially concentrated in the gill and connective tissues prior to spreading systemically after several weeks (Cawthorn, 1997; Athanassopoulou et al., 2004, Greenwood et al., 2005). The systemic spread of *A. haemophila* was accompanied by progressive haemocytopenia (Fig. 3.1). During the early stages of the infection, haemocytes were seen to encapsulate *A. haemophila* in multiple tissues with most encapsulation events occurring in the gills. Even when *A. haemophila* was not visible histologically, there was evidence of haemocyte aggregation and encapsulation that was not seen in control animals (Figs. 2 and 3). Haemocyte mediated encapsulation of *A. haemophilia*, a pathogen that is too large for phagocytosis, is clearly depicted in Fig. 3.3. Haemocytes are initially recruited to *A. haemophila* by some unknown humoral or haemocyte cell surface receptor recognition receptors (Fig. 3.3 A). Subsequent recruitment of granular haemocytes results initially in the surrounding and immobilization of the parasite, and degranulation in the immediate

vicinity of *A. haemophila* (Fig. 3.3 B-D). Degranulation results in the release of toxic metabolites and initiation of phenoloxidase cascades which are capable of killing the immobilized *A. haemophila* (Fig. 3.3 E) (Söderhäll and Cerenius, 1998; Cerenius and Söderhäll, 2004; Cerenius et al., 2008). As the infection progressed, the absence of visible encapsulation events corresponded with haemocytopenia and an increase in the amount of ciliates present in *H. americanus* tissues and circulating freely in the haemolymph.

Microarray analysis identified 926 and 145 *H. americanus* hepatopancreatic genes that are differentially regulated, at $\alpha = 0.05$ and $\alpha = 0.005$ respectively, during the *A. haemophila* disease challenge. One-way ANOVA and HC determined that 145 genes differentially expressed at the $\alpha = 0.005$ level is the most biologically meaningful group of genes for analysis in this study. Although some of these 145 genes may be false-positives, comparison of sample HC generated from these 145 genes to that generated when the 926 genes are subjected to Bonferroni correction, reveals that $\alpha = 0.005$ alone is better able to differentiate infected from control samples (Appendix B Figs. B.1 and B.2). Bonferroni correction is an overly harsh correction method to eliminate false-positives and we believe that there is much more value in retaining true positives at the expense of an increase in false positives. The 38 genes that are found with the Bonferroni correction have an exceptionally high probability of exhibiting true differences in gene expression during *A. haemophila* infection and can be thought of as true-positives. Sample HC based on the expression patterns of the 38 Bonferroni corrected and 145 $\alpha = 0.005$ significant genes represents a meaningful comparison to verify the validity of the $\alpha = 0.005$ cutoff.

Moreover, biologically meaningful information can be gained by increasing the number of identified true-positives, at the expense of including false-positives, if a statistical cutoff of $\alpha = 0.005$ is combined with known or hypothesized biological function. It is more beneficial to start with a larger list of genes that potentially have a role in immunity, and verify their importance through RT-qPCR and bioinformatic rigour, than to limit potential immune genes at this early stage of crustacean immune factor discovery.

K-means clustering is able to reduce the complexity of the 145 differentially expressed genes by clustering them within similar expression profiles (Fig. 3.4 and Appendix B Table B.2). Clustering genes with similar expression profiles highlights the fact that a gene's expression is not independent, and genes under similar expression control are more likely to have similar physiological function (Eisen et al., 1998). Hierarchical Clustering of the samples, or treatments, from these gene clusters adds another level of power to this analysis where assignment of putative physiological function to genes within clusters can be made as the time or condition when they are differentially expressed demonstrates under which physiological processes their expression is important. This is especially valuable as 47.6% of the 145 differentially expressed genes have no known similarity to proteins in GenBank.

Cluster 3 contains 20 genes, including several that have been implicated in crustacean immunity such as three haemocyanins, 2 pseudohaemocyanins, a C-type lectin receptor, VOM-I and Hsp70 (Appendix B Table B.2). These genes are upregulated early in the infection and then decrease to the levels of their respective controls by the end of the trial.

The sample HC of cluster 3 found two main clusters where samples from 5-10 weeks cluster together and samples from 24 h to 5 weeks cluster together. This demonstrates that there is a time-dependent effect that may not involve infection status. Cluster 3 probably represents genes that may be involved only early in the infection and then represents expression indicative of time under reduced caloric intake due to the length of the trial.

Cluster 6 contains 30 genes including PEPCK-1. Sample HC finds three clusters where 24 h and 8 week control samples cluster together, 10 week control, 1 week and 8 week infected samples cluster together and 48 h, 24 h, 3-4 weeks, 5-7 weeks and 10 week infected samples cluster together. This appears to be a separation based on infection. Cluster 6 includes genes that are not recognized as immune genes but perhaps are metabolic genes that are increased early in the infection to try to help *H. americanus* combat the infection. These genes are likely not directly related to mediating immunity, but have an important secondary role.

The sample HC of cluster 7 contains 18 genes including four well known immune genes: ALFHa-4, ALFHa-6, SAA and a Toll-like receptor. We can suggest that all genes in this cluster have some primary function in the *H. americanus* response to *A. haemophila* infection because the samples largely cluster into early infection, late infection and 24 h and 8 week controls. This suggests that they have functions that depend on the stage of *A. haemophila* infection where *A. haemophila* tends to remain largely in the connective tissues for 1-4 weeks prior to progressing into a systemic infection as we, and others, have observed (Athanassopoulou et al., 2004, Greenwood et al., 2005). The assignment of a

putative biological function to cluster 7 is especially valuable to the one third of genes in this cluster that have no known function or meaningful similarity to proteins in GenBank.

The other clusters in this analysis have sample HC that indicate that they may not be involved in the *H. americanus* immune response but rather are a direct reflection of the length of time the lobsters were maintained in holding during the trial. Several recognized immune genes are within these clusters including: thioredoxin 2, DnaJ (Hsp 40), phagocyte signaling impaired protein (PSIP) and a serine proteinase inhibitor.

To verify our microarray findings we chose 5 genes that were differentially regulated at $\alpha = 0.005$ to be evaluated by RT-qPCR. The microarray and RT-qPCR findings in this study show a high level of agreement with an average Spearman's Rho of 0.862. Five additional genes were chosen, four which were differentially expressed at $\alpha = 0.05$, and one which was not differentially expressed, but all have previously been demonstrated to be involved in *H. americanus* immune response (Chapter 2).

Anti-lipopolysaccharide factors are anti-microbial peptides that are known to function in crustaceans, with several identified in *H. americanus* and are thus denoted ALFHa (Beale et al., 2008; Hauton, 2012; Chapter 2). Our microarray measures the expression of 6 ALFHa isoforms, two of which were found to be differentially expressed at $\alpha = 0.005$ and two additional isoforms were differentially expressed at $\alpha = 0.05$. ALFHa-1, ALFHa-2, ALFHa-4 and ALFHa-6 have been shown to be involved in the *H. americanus* response to the Gram-positive lobster pathogen *A. viridans* var. *homari* (Chapter 2). In this study,

RT-qPCR analysis confirmed the role of ALFHa-1, ALFHa-2 and ALFHa-4 in *H. americanus* immune response to *A. haemophila*. Additionally, ALFHa-6 involvement in *A. haemophila* infection was confirmed by microarray but was not analyzed by RT-qPCR. ALFHa-2 expression peaked at 10 weeks in infected animals at 11.52-fold that of the 24 h control and 35.56-fold that of its corresponding time point control (Table 3.1). ALFHa-1 and ALFHa-4 expression was less pronounced and peaked at 5-7 weeks and 10 weeks respectively. The increasing expression of the ALFHa antimicrobial peptide isoforms during the infection coincided with increasing numbers of *A. haemophila* parasites visible during histological analysis. This is suggestive of an attempt by the *H. americanus* immune system to combat an increasingly severe infection.

ALFHa-2 has the highest ALFHa expression during *A. haemophila* infection. In *A. viridans* infections, ALFHa-4 and ALFHa-2 expression peaked at the end of the trial at 26.70-fold and 13.37-fold higher than its corresponding time-point control. This suggests that ALFHa-4 plays a more important role in responding to Gram-positive infections while ALFHa-2 is more important during response to ciliated protist infections. Interestingly, ALFHa-3 and ALFHa-7 were not differentially expressed in response to either *A. haemophila* or *A. viridans* and therefore may represent another *H. americanus* pathogen or pathogen-class specific immune response that has not yet been evaluated.

Acute phase serum amyloid protein A was found to be significantly upregulated in both our microarray and RT-qPCR analysis, where its expression peaked at the end of the 10 week trial in infected animals at 54.95-fold higher than its time-point control (Table 3.1).

Acute phase serum amyloid protein A expression was not statistically different than the 24 h control at 24 h and 48 h in infected animals, but it steadily increased over the length of the trial to 22.14-fold higher than the 24 h control by 10 weeks. Acute phase serum amyloid protein A is especially interesting because it is a widely known acute phase protein and an indicator of inflammation and innate immune response in many vertebrate systems (Cray et al., 2009). To our knowledge, SAA has not been used as an indicator of an activated immune system in crustaceans. We have previously shown that SAA expression peaks at 30.64-fold its time-point control when lobsters become moribund due to *A. viridans* infection (Chapter 2). Coupled together, our current findings suggest that SAA may be a good marker of immune activity or disease status in *H. americanus* and potentially in crustaceans in general. Increased expression of hepatic SAA has also been found in carp *Cyprinus carpio* during infection with *Ichthyophthirus multifiliis*, demonstrating a common increase in APP in response to a ciliate infection (Gonzalez et al., 2007).

C-type lectin receptor expression is initially upregulated 5.06-fold at 24 h during infection but decreased by 48 h and remained low until the end of the 10 week study (Table 3.1). Lectins have an important role during innate immunity in both vertebrates and invertebrates, where they bind carbohydrate motifs and can act as agglutinins and opsonins, while some play a role in activating the prophenoloxidase activating system (Marques et al., 2000; Lee and Söderhäll, 2002). Interestingly, the increased expression of a C-type lectin receptor in the hepatopancreas occurs even when no *A. haemophila* were found in the hepatopancreas. The hepatopancreas is the location of fixed phagocytes that

are responsible for removing bacteria, viruses and other small foreign particles from the systemic circulatory system (Johnson et al., 1981) but *A. haemophila* is too large to be phagocytosed. If the lectin receptors are released into the circulating haemolymph then perhaps they bind to lectins or carbohydrates on the surface of pathogens, or perhaps to *H. americanus* lectins already bound to *A. haemophila*. This binding could increase the signal for circulating haemocytes to aggregate near *A. haemophila* and initiate the prophenoloxidase-based encapsulation process. Encapsulation, and the expression of this C-type lectin receptor, is only observed early in infection. Further examination of the function of this C-type lectin will be required to validate whether encapsulation is facilitated by this C-type lectin receptor or if its temporal expression and encapsulation events is coincidental. A similar small induction and subsequent decrease in expression was seen for a C-type lectin in *Cyprinus carpio* during *I. multifiliis* infection (Gonzalez et al., 2007).

Pseudohaemocyanin-2 precursor (PHc-2), sometimes called cryptocyanin, is related to haemocyanin except that it does not bind copper and is considered a storage protein (Burmester, 1999; Terwilliger et al., 1999). Except for a slight increase at 48 h, PHc-2 expression steadily decreased to a 10-fold down regulation by 10 weeks in both infected and control samples (Table 3.1). This decreased expression is likely indicative of the long time in holding and not due to infection, as the control lobsters show similar expression at their respective time-points. Pseudohaemocyanin-2 precursor is in cluster 3 where a sample HC of this cluster separate into before or after 5 weeks in the trial irrespective of infection status, providing additional evidence that this gene is a marker of time in the

trial and not infection status. Since tens of millions of live lobsters are kept in storage every year for differing lengths of time to meet commercial market demands, it would be interesting to see if PHc-2 could be a marker of storage length or prolonged decrease in nutritional intake.

Phosphoenolpyruvate carboxykinase 1 is the rate-limiting step in gluconeogenesis. There is an approximately 3-fold downregulation in expression of PEPCK-1 in infected lobsters from the early points (24 h and 48 h) in the trial to week 10 (Table 3.1). The expression level is never different between an infected sample group and its time-point control. PEPCK-1 belongs to cluster 6 and the sample HC of this cluster indicates that that there is a separation between the 24 h and 8 week controls and the rest of the samples. The 24 h control is not significantly different than the 8 week and 10 week controls but the 24 h and 48 h infected animals are significantly different than the infected animals after 3 weeks, with the exception of weeks 5-7 for 48 h. This indicates that the infected samples have higher expression early but decline over the course of the trial, whereas the control lobsters do not. The higher PEPCK-1 expression early in the trial by infected lobsters is not due to any stress that could have been caused by the initial injection, as there is no difference in PEPCK-1 expression in control animals across all time-points. Another possibility is that *H. americanus* can sense the initial *A. haemophila* infection and respond to this stress by increasing gluconeogenesis but cannot sustain an increase in glucose production due to low nutrient availability or glycogen stores. This increased gluconeogenesis could provide the additional energy required for immune response in addition to physiologically homeostatic processes.

Trypsin-1a has an initial increase in expression over all of the infected and control time-points, with the exception of 8 week infected samples. This is a difference of 2.69- and 3.58-fold for the expression at 24 h over the 10 week infected and 8 week control respectively (Table 3.1). None of the control samples significantly differ from each other. Trypsin-1a expression is similar to PEPCK-1 and therefore may be due to increased stress/metabolic demand during the initial *A. haemophila* infection. Trypsin-1b was found to be significantly differentially expressed via microarray at the $\alpha = 0.005$ level, and the RT-qPCR data agree very well with a Spearman's Rho of 0.952 (Table 3.1). There is a slight increase in expression in the 5-7 week and 8 week infected animals but this is not statistically different than the 8 week control even at 8.85- and 12.58-fold greater expression. However, the expression of trypsin-1b at 10 weeks is significantly different when compared to its time-point control with a 10.80-fold increase. This is in contrast to tryspin-1a where the early time point infected 24 h samples are higher and both control and infected decrease over the trial.

Trypsin-1a and 1b are serine proteases and these types of proteases play a significant role in mediating innate immunity signalling cascades in crustaceans (Cerenius and Söderhäll, 2004). Four trypsin-like genes have been found to be upregulated in the Chinese shrimp *Fenneropenaeus chinensis* during White Spot Syndrome Virus (WSSV) infection and one was upregulated during a Gram-negative *Vibrio anguillarum* bacterial challenge (Shi et al., 2009). Trypsins also act as digestive enzymes and are members of multi-gene families found in both invertebrate and vertebrate systems (Klein et al., 1998). So whether these

trypsin or trypsin-like gene expression changes play a metabolic, immune or combination role remains to be found.

Trypsin-1b was found to be upregulated almost 70-fold in *A. viridans* infected lobster while trypsin-1a expression was unchanged (Chapter 2). The over-expression of Trypsin-1b in both *A. haemophila* and *A. viridans* infections presents a compelling case for its role as an immune factor in *H. americanus*.

Thioredoxin is a redox protein that functions in defence of oxidative stress, such as neutralization of reactive oxygen species. It can act intracellularly, or can be secreted where it has co-cytokine and chemokine activities (Arner and Holmgren, 2000). Although we found that there was very little agreement between the microarray and RT-qPCR data based on a Spearman's Rho 0.212, RT-qPCR confirmed that thioredoxin is not significantly differentially regulated during *A. haemophila* infection (Table 3.1). The lack of agreement between the RT-qPCR and microarray data is likely due to the combination of small inter-treatment difference in expression and moderate intra-treatment expression variation. Thioredoxin has previously been found to be involved in the *H. americanus* response to *A. viridans* where its expression was increased over 5-fold in moribund infected lobsters (Chapter 2). This lack of differential expression could be due to the fact that *A. viridans* accumulates in the fixed phagocytes of the hepatopancreas where they replicate and eventually rupture the cell releasing its known ROS contents (Moss and Allam, 2006). In *A. haemophila* infections the fixed phagocytes remain intact as *A. haemophila* are not found in the hepatopancreas.

3.6 Conclusion

One hundred and forty-five genes were found to be differentially expressed in *H. americanus* during *A. haemophila* infection. Although not all of these genes will have a primary role in immune response, some of these genes are clearly emerging as having defined roles in *H. americanus* immunity and many are noted for the first time. It is becoming clear that ALFHa isoforms have a strong role in *H. americanus* immune response where differential expression can be seen for different pathogens. Another interesting finding is the large increase in SAA during pathogen infection. The use of serum acute phase proteins are already widespread in veterinary and human medicine (Cray et al., 2009) and its utility in crustacean disease or health monitoring could prove to be very valuable.

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Supplementary data associated with this article can be found in Appendix B.

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4. Molecular immune response of the American lobster (*Homarus americanus*) to the White Spot Syndrome Virus

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K.F. Clark designed and carried out the experiments, analyzed the results and wrote the manuscript. S.J. Greenwood supervised the work and writing and A.R. Acorn provided technical assistance. P.J. Byrne provided and supervised the specialized exotic pathogen holding facilities, analyzed the histology, and supervised the work and writing.

4.1 Abstract

The adult American lobster (*Homarus americanus*) is susceptible to few naturally occurring pathogens, and no viral pathogen is known to exist. Despite this, relatively little is known about the *H. americanus* immune system and nothing is known about its potential viral immune response. Hundreds of rural communities in Atlantic Canada rely on the lobster fishery for their economic sustainability and could be devastated by large-scale pathogen-mediated mortality events. The White Spot Syndrome Virus is the most economically devastating viral pathogen to global shrimp aquaculture production and has been proposed to be capable of infecting all decapod crustaceans including the European Lobster. An *in vivo* WSSV injection challenge was conducted in *H. americanus* and WSSV was found to be capable of infecting and replicating within lobsters held at 20 ^{o}C. The *in vivo* WSSV challenge also generated the first viral disease model of *H. americanus* and allowed for the high-throughput examination of transcriptomic changes that occur during viral infection. Microarray analysis found 136 differentially expressed genes and the expression of a subset of these genes was verified using RT-qPCR. Anti-

lipopolysaccharide isoforms and acute phase serum amyloid protein A expression did not change during WSSV infection, contrary to previous findings during bacterial and parasitic infection of *H. americanus*. This, along with the differential gene expression of thioredoxin and trypsin isoforms, provides compelling evidence that *H. americanus* is capable of mounting an immune response specific to infection by different pathogen classes.

4.2 Introduction

The American lobster (*Homarus americanus*) fishery is the most significant commercial fishery in Canada (Gardner Pinfold, 2010). Adult lobsters have few known naturally occurring pathogens, none of which are viral (Cawthorn, 2011). The European Union EC Directive 2006/88/EC lists all decapod crustaceans (>20,000) as susceptible to White Spot Syndrome Virus (WSSV) even though scientific data supporting this susceptibility exists for less than 1% of decapods (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:en:PDF). White Spot Syndrome Virus initially emerged in Southeast Asia in the early 1990s, and has since spread to most shrimp aquaculture countries in Asia, North, Central and South America as well as the Middle East (Walker and Mohan, 2009; Lightner and Redman, 2010; Sánchez-Paz, 2010). White Spot Syndrome Virus is the most significant impediment to the growth and sustainability of the global shrimp aquaculture industry and has caused close to $10 billion in lost productivity since its emergence (Stentiford et al., 2012). Although WSSV has been discovered in wild shrimp caught on the Southeast coast

of the U.S.A. (Chapman et al., 2004), WSSV has never been detected in crustacean species originating from Canada, or in *H. americanus* in the U.S.A.

The molecular mechanisms that *H. americanus* utilizes to respond to pathogens are only beginning to be understood. The use of high-throughput transcriptomic analysis of *H. americanus* hepatopancreatic tissue during Gram-positive bacterial infection with *Aerococcus viridans* var. *homari* or the scuticociliate *Anophryoides haemophila* has highlighted several novel genetic mediators of immunity (Chapter 2 and 3). Many of the genes highlighted in these studies have functions that were previously unknown, and have no similarity to proteins in GenBank. Some of the key findings were the importance of *H. americanus* anti-lipopolysaccharide factors (ALFHa) and the differential expression of ALFHa isoforms within and between bacterial and parasitic infections (Beale et al., 2008; Chapter 2 and 3). Acute phase serum amyloid protein A (SAA) was also found to play a role in *H. americanus* immune response as it was found to be greatly increased in moribund animals (Chapter 2 and 3). Other studies have found a small number of *H. americanus* antimicrobial peptides (Battison et al., 2008), plasma lectins (Battison and Summerfield, 2009) a crustin (Christie et al., 2007) and haemolymph based agglutinin factors (Cornick and Stewart, 1973; Hall and Rowlands, 1974a; 1974b; Hartman et al., 1978; Abel et al., 1984) but there remains no information on how *H. americanus* responds to viral infections.

Although there is no known viral pathogen of *H. americanus*, regulatory investigations being carried out into the potential susceptibility to WSSV presented an opportunity to

generate a viral model of disease. This viral model could then be used to study the immune response of *H. americanus* to a viral pathogen and compare to *H. americanus* responses to bacterial and parasitic pathogens (Chapter 2 and 3). The aim of this study was to challenge *H. americanus* with WSSV and monitor the transcriptomic changes that occur in hepatopancreatic tissue. The hepatopancreas is the major humoral immune organ in *H. americanus* (Factor and Beekman, 1990) and presents an excellent opportunity for the discovery of novel *H. americanus* immune factors using a high-throughput microarray approach. In addition, the susceptibility of *H. americanus* to WSSV infection was monitored with a variety of clinical, molecular and histological approaches to evaluate the progression of WSSV infection in *H. americanus.*

4.3. Materials and Methods

4.3.1 Animal Handling

Adult male American lobsters, *H. americanus*, (n = 31, 578.0 g ± 17.3 g) were purchased from a local seafood supplier (Bedford, Nova Scotia) and were assessed for physical and clinical indicators of health (Acorn et al., 2011). Health assessments included bacterial and ciliated protist infection screening of the haemolymph, haemolymph refractive index measurement of total dissolved solutes, total haemocyte counts (THC) and qPCR testing of haemolymph and pleopods tissues for WSSV. Animals were housed, and pathogen challenges carried out, at the Charlottetown Aquatic Animal Pathogen Biocontainment Laboratory, Department of Fisheries and Oceans in Charlottetown, Prince Edward Island. Lobsters were handled in accordance with the University of Prince Edward Island Animal Care Committee approved animal care protocol #09-052 and the Department of Fisheries

and Oceans Regional Animal Care Committee approved animal use protocols 09-23 & 10-13. Lobsters were housed in individual compartments of perforated plastic totes capable of housing six lobsters per tote. Two storage totes were placed in each of three 400 L tanks. Each tank was supported by an independent recirculating artificial seawater (ASW) system (Instant Ocean, 30 ppt) equipped with 25 µm mechanical filter, UV filtration, foam fractionation and 10-15% water replacement per day. Lobsters were acclimated to 20 °C by increasing the water temperature from 8 °C in 1.5 °C per day increments to reach the minimum water temperature where natural WSSV infections of *Litopenaeus vannamei* usually occur (Guan et al., 2003). Temperature was maintained at 20 ± 0.2 °C. Water quality parameters including pH, oxygen, ammonia and nitrite were monitored weekly. Lobsters were held for two weeks prior to the beginning of the experiment, which included the acclimation period, without feeding.

Waste material was sterilized prior to disposal; carcasses were incinerated, water effluent autoclaved and washing debris, laboratory disposable waste and clothing autoclaved. Work with live WSSV was approved through permits from the Canadian Food Inspection Agency, Biohazard Containment and Safety section.

4.3.2 White Spot Syndrome Virus inoculations

Frozen WSSV-infected shrimp were obtained from Dr. Donald Lightner at the OIE reference laboratory for WSSV at the University of Arizona. Shrimp were infected with WSSV isolate (AF332093), originally isolated from an outbreak in China. Muscle tissue from infected shrimp was homogenized and diluted in phosphate buffered saline. Shrimp

homogenate was quantified against WSSV plasmid standards using a quantitative PCR (qPCR) test (see section 4.3.9) and diluted to the equivalent of 10^4 plasmid copies/µL.

4.3.3 WSSV Infection Challenge

Lobsters were randomly assigned to receive either 200 µL injections of 10^4 WSSV genome copies/µL (n = 24) or sterile 3% NaCl (n = 3). Shrimp homogenate containing WSSV was 0.45 µm filtered, aliquoted into syringes and then stored on ice (45 minutes) until inoculation. White Spot Syndrome Virus challenged lobsters were randomly selected, prior to the start of the challenge, for lethal sampling (n = 4) at 6 h, 12 h, 24 h, 48 h, 96 h and 168 h and control lobsters were randomly selected for sampling at 6 h (n = 3) and 168 h (n = 4). The 168 h final sampling time was chosen because most of the remaining WSSV infected lobsters were moribund based on THC and physical assessments for vigour and behaviour. Lobsters were not fed during the infection challenge.

4.3.4 *H. americanus* Sampling

Haemolymph sampling was conducted to determine the concentration of total circulating haemocytes, and to screen for the presence of bacteria, as described in Battison et al. (2003), and ciliated protists (Acorn et al., 2011). *H. americanus* were lethally sampled by severing the ventral nerve cord anterior to the cheliped as per Chapter 2. Hepatopancreatic tissue was taken for RNA analysis while 12 tissues were collected for histology and qPCR based detection of WSSV including: haemolymph, hepatopancreas,

heart, gill, antennal gland, testis, stomach, intestine, ventral nerve cord, cuticular epithelium, tail muscle and claw muscle.

Hepatopancreatic tissue was preserved for microarray and RT-qPCR analysis by immediate homogenization in RNA preservation reagent (1.4M guanidine isothiocyanate, 38% phenol (pH 4), 5% glycerol and 0.1M sodium acetate). Homogenized tissue was flash frozen in a dry ice-ethanol bath and stored at -80 °C.

RNA was isolated as previously described (Chapter 2). Briefly, chloroform was added to thawed tissue homogenate (200 µL/mL RNA preservation reagent), shaken, incubated at room temperature and then centrifuged at 12,000*g* for 15 min at 4 °C. Equal volumes of the resulting supernatant was mixed with 70% ethanol, and added to an RNeasy spin column (Qiagen, Toronto, ON , Canada). RNA was isolated following the manufacturer's instructions including the optional DNase I (Qiagen) treatment. Total RNA was quantified using a NanoDrop-1000 (Thermo Fisher, Ottawa, ON, Canada) and the RNA quality was assessed using an Agilent Bioanalyzer (Agilent, Mississauga, ON, Canada). RNA samples were stored at -80 °C prior to use.

Tissues for histology were fixed in 10% formalin in 0.2 µm filtered ASW, dehydrated and infused with paraffin wax using an infiltration processor and standard protocols. A rotary microtome was used to cut serial sections (3 to 5 µm) which were mounted on glass slides. Sections were stained with haematoxylin and eosin (H & E) and evaluated using a light microscope (Zeiss Axioplan 2).

Tissues for electron microscopy were incubated in 2 % glutaraldehyde in 0.1 M phosphate buffer overnight at 4 ^{o}C, washed with 0.1 M phosphate buffer and then post-fixed in 1% OsO_4 in phosphate buffer overnight at 4 ^{o}C. Tissues were dehydrated in progressively more concentrated ethanol solutions and infiltrated with Epon with propylene oxide. Ultra-thin samples were viewed on a Hitachi H-7500 transmission electron microscope and images were captured with an HR CCD AMT digital camera.

4.3.5 *H. americanus* Microarray Design and Construction

The *H. americanus* microarray used has previously been described in detail (Chapter 2). The array consists of 14,592 50 mer DNA probes and 784 control features designed from *H. americanus* EST and nucleotide sequences in GenBank.

4.3.6 cDNA Labeling and Microarray Hybridization

A SuperScript™ Plus Indirect cDNA Labeling System (Life Technologies Inc., Burlington, ON, Canada) was used to label single strand cDNA, generated from 20 µg high quality sample RNA with Alexa Fluor® 555. Reference aRNA was generated from 5 µg of pooled lobster hepatopancreatic RNA using an Amino Allyl MessageAmp™ II aRNA Amplification kit (Life Technologies Inc.). Reference aRNA was labeled with Alexa Fluor® 647 using 12 µg of aRNA and a Superscript™ Plus Indirect cDNA Labeling System (Life Technologies Inc.). Labeled aRNA and cDNA (100 pmol and140 ng) were quantified using a NanoDrop 1000 and used for microarray hybridization. Labeled aRNA was fragmented using 1 µL 10x Fragmentation solution and incubation for

10 min at 70 °C followed by 1 µL of Stop solution (Life Technologies Inc.). Hybridizations were performed using A2 chambers on a Tecan 400 HS Pro hybridization system (Tecan, NC, USA) as previously described (Chapter 2).

4.3.7 Microarray Data Analysis

Microarrays were imaged using a GenePix 4000B scanner (Molecular Devices, PA, USA), features were extracted using SpotReader v 1.3.1 (Niles Scientific, CA, USA) and resulting data was analyzed using TM4/MeV v4.8.1 (Saeed et al., 2003). MIDAS (Microarray Data Analysis Software) v2.2.2 was used for flagging features and features were normalized using LOWESS (Quackenbush, 2002). Log2 expression ratios of sample to reference gene intensity was generated to compare arrays with one way ANOVA analysis (MeV) at $\alpha = 0.05$ with and without Bonferroni correction and at $\alpha = 0.005$ without Bonferroni correction, all with 1000 permutations. Figure of Merit (FOM), K-means clustering and Hierarchical clustering (HC) were used to determine similarity between the expression profiles of significantly differentially expressed genes (Soukas et al., 2000; Yeung et al., 2001). Raw and normalized microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GSE45742 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45742 .

4.3.8 Verification of Microarray Results

Microarray findings were verified using reverse transcription quantitative polymerase chain reactions (RT-qPCR) to measure the mRNA expression of 14 selected genes of

interest: eukaryotic translation initiation factor 3 subunit 5 epsilon-like (eTIF3-5e), cathepsin C1, cathepsin C2, cathepsin C3, heat shock protein 21 (Hsp 21), ecdysone-inducible protein E75 (EIPE75), phosphoenolpyruvate carboxykinase 1 (PEPCK-1), trypsin-1a, trypsin-1b and thioredoxin. *H. americanus* anti-lipopolysaccharide factors (ALFHa-1, ALFHa-2, ALFHa-4), and acute phase serum amyloid protein A (SAA) (Table 4.1). Normalization genes were validated using geNorm^{PLUS} in the qbase^{PLUS} qPCR analysis software suite v2.3 (Biogazelle, Belgium). A total of 3 genes (conserved hypothetical protein, WD repeat protein 26 and microtubule-associated protein RP\EB family member 3) were determined to be the minimal number of normalization candidates required for analysis (Table 4.1).

RT-qPCR was performed as previously described (Chapter 2). Briefly, cDNA was generated from 1 µg of high quality sample total RNA using the Superscript III First Strand Synthesis kit (Life Technologies Inc.), as per the manufacturer's instructions. qPCR reactions were performed using 2 µL of diluted cDNA in a total reaction volume of 15 µL using a Chromo4™ Real-Time PCR system (BioRad, Hercules, CA). qPCRs contained 1x Express SYBR® GreenER™ with ROX (Life Technologies Inc.) and 200 nM of each primer (Table 4.1). All qPCR were set up in triplicate by a QIAgility (Qiagen) liquid handling robot to improve precision and reproducibility. All qPCR reactions were optimized for temperature and amplicons were run on 2% agarose gels to confirm single amplicon of the proper size.

Table 4.1 Forward and reverse primers used in RT-qPCR experiments.

Gene	Accession #	Forward	Reverse	Annealing Temperature (°C)	Reaction Efficiency (%)	# Step Reaction
Eukaryotic Translation Initiation Factor 3-5e	EH035483	GGCCAAGTTCATCCTGTGGTGTTT	TCAATGGCTCCCTTATCCACACCT	65	102	3
Cathepsin C1	EH116404	AAGAACTTCACCCTCAGCTTCCCA	ATCACAGTAGGACACCACAGCGTT	65	102	3
Cathepsin C2	EV782128	CGTCGCCAAAGATGAGCTGAACAA	AAGAAACCATGTCGCCCTCGTAGT	65	92	3
Cathepsin C3	EH116090	ACCACTCGTTTGTGGAAACCAAGC	ATAATCCAGTCACCACGCACGTCA	65	91	3
Ecdysone-inducible protein E75	EX471223	CACCAAGAACCAACAGTGCTCCAT	ATCTCTGGACATGCCGACAGCAAT	62.5	93	2
Thioredoxin	FD699182	TTTGACAAGCAGTTGGCTGATGCC	TCCACGTCCACCTTCAAGAACACT	65	93	3
Trypsin 1a	EV781656	CGTCCAATGTTAAGCGTCATGCCA	TTCCAAGTCTTGCCCGTAGACACA	65	93	3
Trypsin 1b	EF095144	AGCTACCGCAACATCGGCTATACT	ATGAAGTAGCGGTTGTCAGCTCCA	67	92	3
Hsp 21	DV774085	TCAGTGCAGCAGCAATCTTCCTCA	AACTCTTGTTGTTGGCGGAGAAGC	62.5	91	2
Phosphoenolpyruvate carboxykinase 1	DV773064	TTGGTTCCAACATTGCCAGACGTG	AAGCGATATCATCACCCACGCACT	64	99	3
ALFHa-1	EU625516	CAGTCGTTCTGGTGTTGTTGGGAA	TTGTTGGGCATCCCTCTCGGTTAT	65	99	3
ALFHa-2	FC556430	AGACTACCACTGACTTCGTGAGGA	TCTCGGGATGATCCGTTAACACCT	65	95	3
ALFHa-4	DV772634	ACAAGACAAGAGAATGCGTCCCTC	TGATAGCTTGTCCACGAAGGCTG	65	93	3
SAA	EH116055	TACCACTACCAGCACTCATCACCT	TCAAACACAGAGAATAGGCACGGG	59	99	3
Conserved hypothetical protein	FE659358	TCAAGCCTGAAGCTGGGATATGCT	AAACACATGGGTTGGATGGCACAG	66.5	92	2
WD repeat protein 26	FF277218	GTGTTCATTTGTGGGACCTACGCA	TGTCTTCACTTCCAGATGCCACGA	57	101	2
Microtubule-associated protein RP\EB family member 3	EX827404	CAAGATCTTGCAGTATGCCTT	TGAACCACTGAAGGAATTCA	65	100	3

Two-step and three-step qPCR reactions were used in order to maximize reaction efficiency and reproducibility. Two-step qPCR protocols were performed as follows: 50 °C for 2 min 95 °C for 2 min followed by 39 cycles of 95 °C for 7 s then 20 s at the gene-specific annealing temperature (Table 4.1) followed by a plate read. Three-step qPCR protocols were the same as two-step reactions with the addition of 72 °C for 20 s after each annealing step. Product specificity was assessed during every reaction with melt curves (65 - 90 °C in 1 °C increments every 2 s) to ensure single peaks. All samples were run in triplicate and every gene run contained negative RT and negative template controls to confirm the absence of contaminating genomic DNA.

4.3.9 qPCR Based Detection of WSSV

Detection of WSSV was performed on DNA extracted from shrimp or lobster tissue using a Qiagen DNeasy kit according to the manufacturer's instructions. To minimize the possibility of cross reactivity with *H. americanus* genomic DNA, primers were developed targeting the VP28 gene of WSSV: forward primer F1-WSDvp28 (5'GTGACCAAGACCATCGAAAC3'), reverse primer R1-WSDvp28 (5'TGAAGTAGCCTGATCCAACC3') and TaqMan probe P1-WSDvp28 (5'CCTCCGCATTCCTGTGACTGC3'-FAM/TAMRA). qPCR reactions consisted of 12.5 µL of ABI TaqMan® Universal PCR Master Mix, 1 µL of DNA extracted from shrimp or lobster tissue, 300 µM forward and reverse primers, 150 µM TaqMan probe and molecular biology grade water up to final reaction volume of 25 µL. Reactions were carried out on a Chromo4™ Real-Time PCR system (BioRad) as follows: 50°C for 2 min,

95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min, plate read, followed by a final incubation at 20°C for 5 min.

4.4 Results

4.4.1 Confirmation of WSSV Infection

Multiple methods were employed to determine the health of the experimental animals throughout the trial. Physical indicators of health including behaviour and strength of defensive posture were combined with clinical indicators of health such as haemolymph refractive index and THC. A combination of THC and behavioural indicators revealed that infected *H. americanus* were moribund at 168 h so the trial was ended. Total haemocyte counts were measured throughout the trial and indicated that infected *H. americanus* circulating haemocyte concentrations decreased during the WSSV challenge and became statistically significantly different from control animals by 168 h (Fig. 4.1). There were no statistically significant differences between haemolymph refractive indices of control and WSSV infected lobsters.

Histology was performed on 11 *H. americanus* tissues including: hepatopancreas, antennal gland, gill, heart, stomach, cuticular epithelium, nerve cord, intestine, tail muscle, claw muscle and testis. Intranuclear inclusions within hypertrophic nuclei of antennal gland tissue was found in all moribund WSSV infected lobsters at 168 h (Fig. 4.2). Electron microscopy on the antennal gland of a moribund WSSV challenged lobster at 168 h confirmed the presence of virions of the known size and shape of WSSV (Lu et al., 1997) (Fig. 4.2).

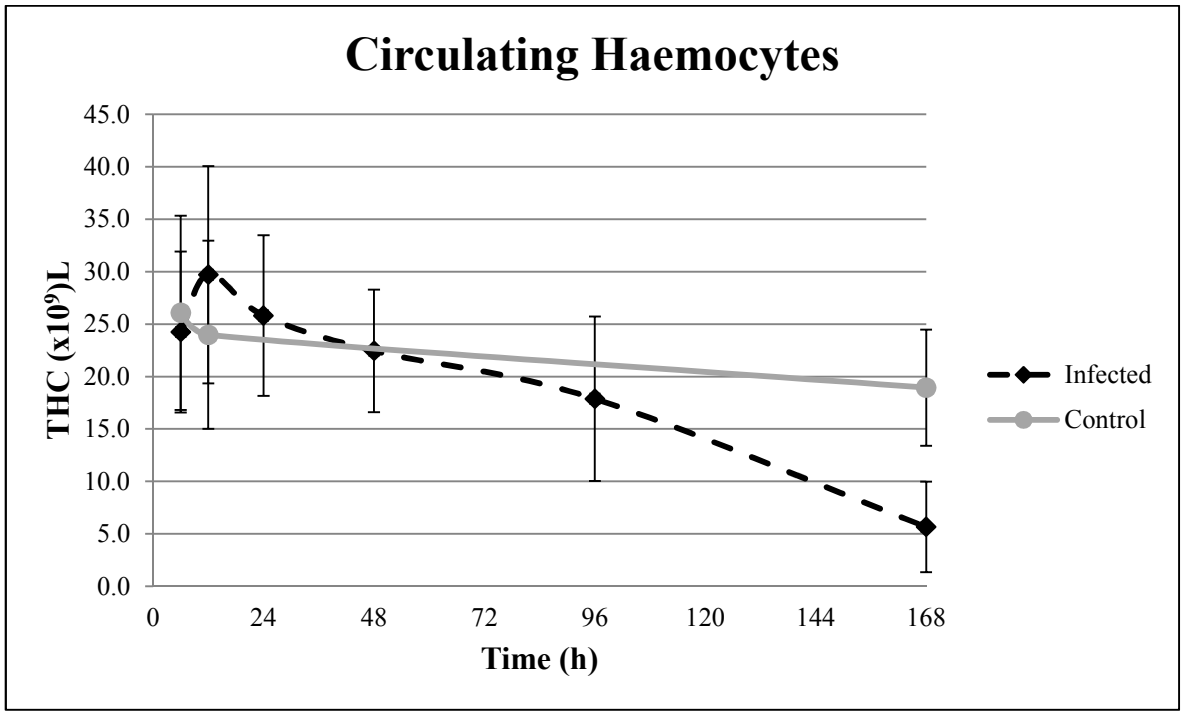

Figure 4.1 *Homarus americanus* total circulating haemocytes during WSSV challenge.

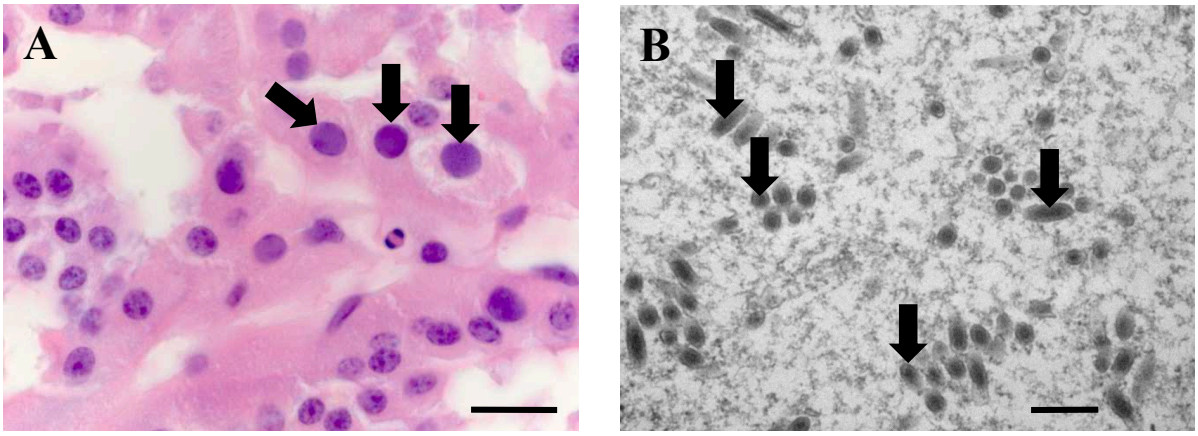

Figure 4.2 *Homarus americanus* antennal gland tissue infected with WSSV. (A) Light microscopy showing hypertrophied nuclei with eosinophilic inclusions (black arrows) stained with H&E. (B) Transmission electron micrograph showing viral particles in the nucleus (black arrows). Bar represents (A) 20 m and (B) 500 nm.

qPCR was performed using primers specific for WSSV to provide additional evidence of the presence and proliferation of WSSV in *H. americanus*. Haemolymph qPCR confirmed the presence of WSSV in infected *H. americanus* and found increasing WSSV concentrations during the trial (Fig. 4.3). All haemolymph samples from control lobsters were negative for WSSV.

4.4.2 Microarray Analysis

Hepatopancreatic tissue gene expression was measured using an *H. americanus* microarray capable of simultaneously monitoring the gene expression of over 14,000 *H. americanus* genes (Chapter 2). One-way ANOVA analysis with a significance cutoff of $\alpha = 0.005$ determined that 136 genes were differentially expressed. Hierarchical Clustering of the samples from these differentially expressed genes results in six clusters where 168 h control samples and 6 h control samples, with the exception of one 6 h control, occupy clusters that do not contain infected samples (Fig. 4.4). The remaining clusters contain samples that are populated mainly by: infected 12 h or 24 h samples, infected 12 h - 48 h samples, infected 48 h – 168 h samples and a final cluster containing one each of 168 h, 96 h and 48 h infected samples, one 6 h control sample and three out of the four 6 h infected samples.

Of the 136 statistically significantly differentially expressed genes found with one-way ANOVA at $\alpha = 0.005$, over 46% were not similar to proteins in GenBank and therefore have unknown functions. Figure of merit (FOM) and K-means clustering was used to cluster these 136 genes based on the similarity of their average treatment gene

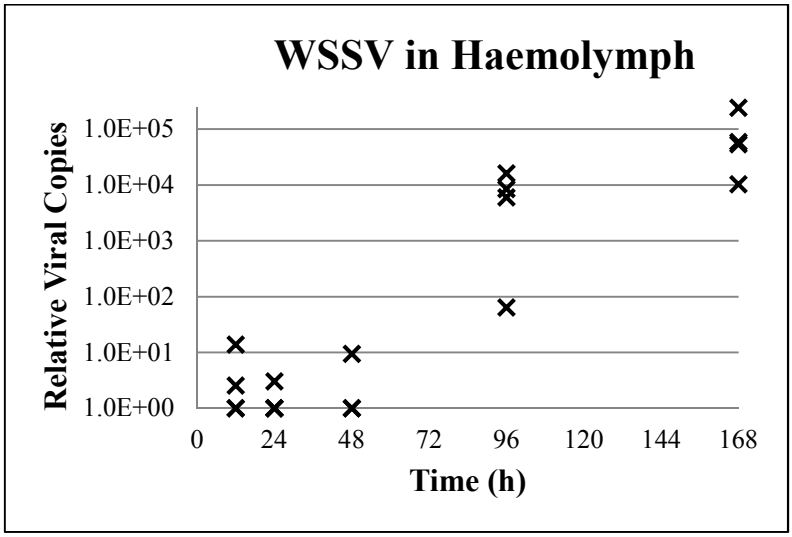

Figure 4.3 WSSV detected by qPCR in lobster haemolymph following injection with WSSV infected shrimp homogenate. Viral copies quantified relative to the concentration of plasmids containing the qPCR amplicon of interest.

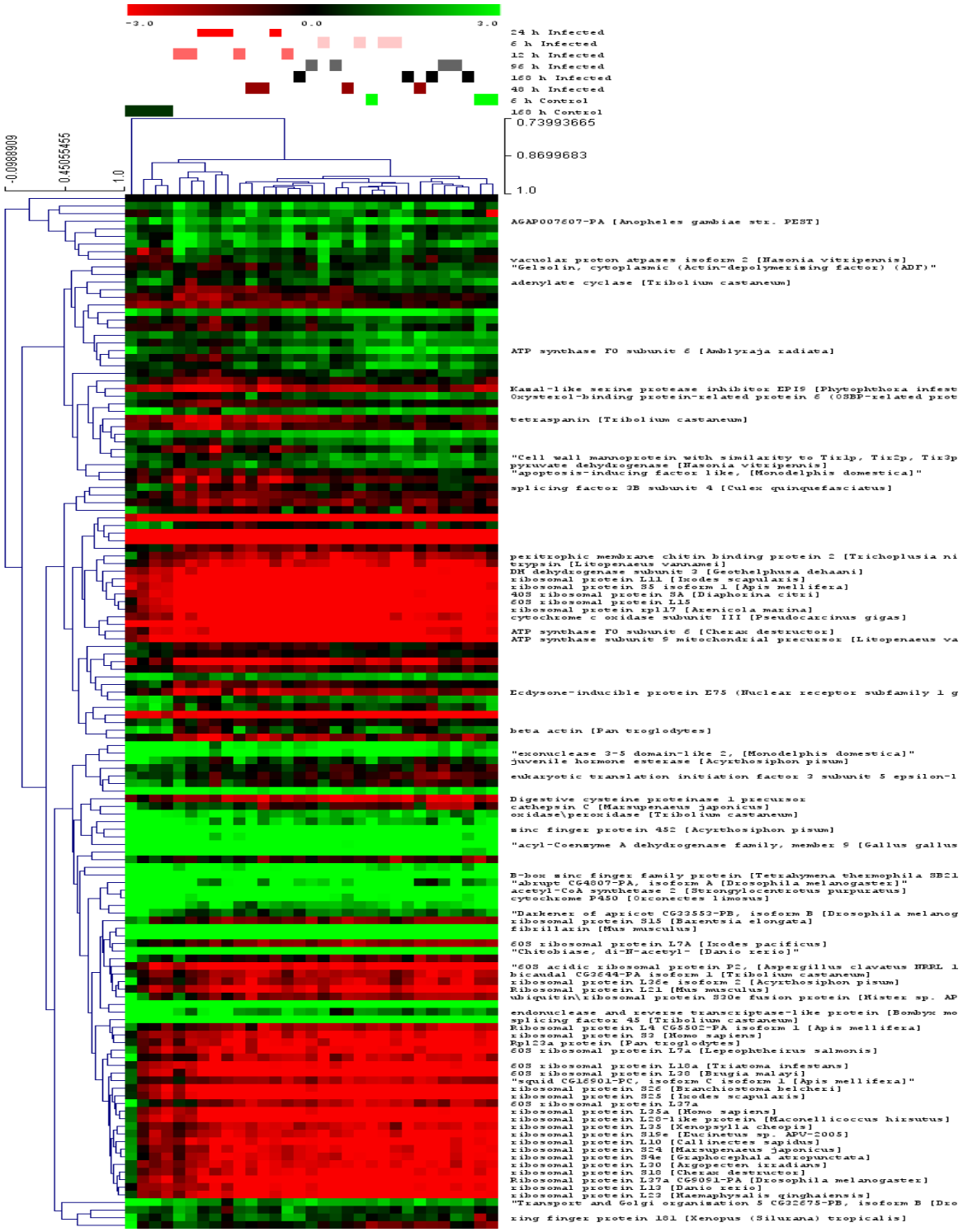

Figure 4.4. Hierarchical clustering of 136 differentially expressed *Homarus americanus* hepatopancreatic genes following WSSV challenge at $\alpha = 0.005$. Gene expression heat maps illustrate the $\log_2$ ratio of experimental sample/reference sample, where a gradient of red to green represents a threefold or greater decrease in gene expression to a threefold or greater increase in gene expression.

expression profiles (Fig. 4.5 and Appendix C Table C.1). The five resulting clusters where then subjected to HC of their samples to see if a gene expression pattern existed that separated the samples either based on time, treatment or a combination of both. Cluster one contained 28 genes whose sample treatments were separated based on time in the trial where 6 h control samples clustered with 6 h and 12 h infected samples, 24 and 48 h infected samples clusters together, 96 h and 168 h infected samples clustered together and 168 h control remained distant from all of the other treatments. Cluster five contained 11 genes with separation of sample treatments based on both infection status and time in infection, where no control sample treatments clustered with infected treatments and infected sample treatments separated into 6 – 24 h infected sample treatments and 48 h - 168 h sample treatments. The remaining three clusters contained genes where no clear link to a primary immune function could be made based on their sample HC.

4.4.3 RT-qPCR Verification of Differential Expression

Several genes were selected to verify the microarray findings of differential expression by analyzing their gene expression with RT-qPCR (Table 4.2). RT-qPCR analysis of gene expression was performed on 14 genes of interest including: eTIF3-5e, cathepsin C1, cathepsin C2, cathepsin C3, ecdysone-inducible protein E75, thioredoxin, trypsin 1a, trypsin 1b, Heat shock protein (Hsp) 21, PEPCK, ALFHa-1, ALFHa-2, ALFHa-4 and SAA.

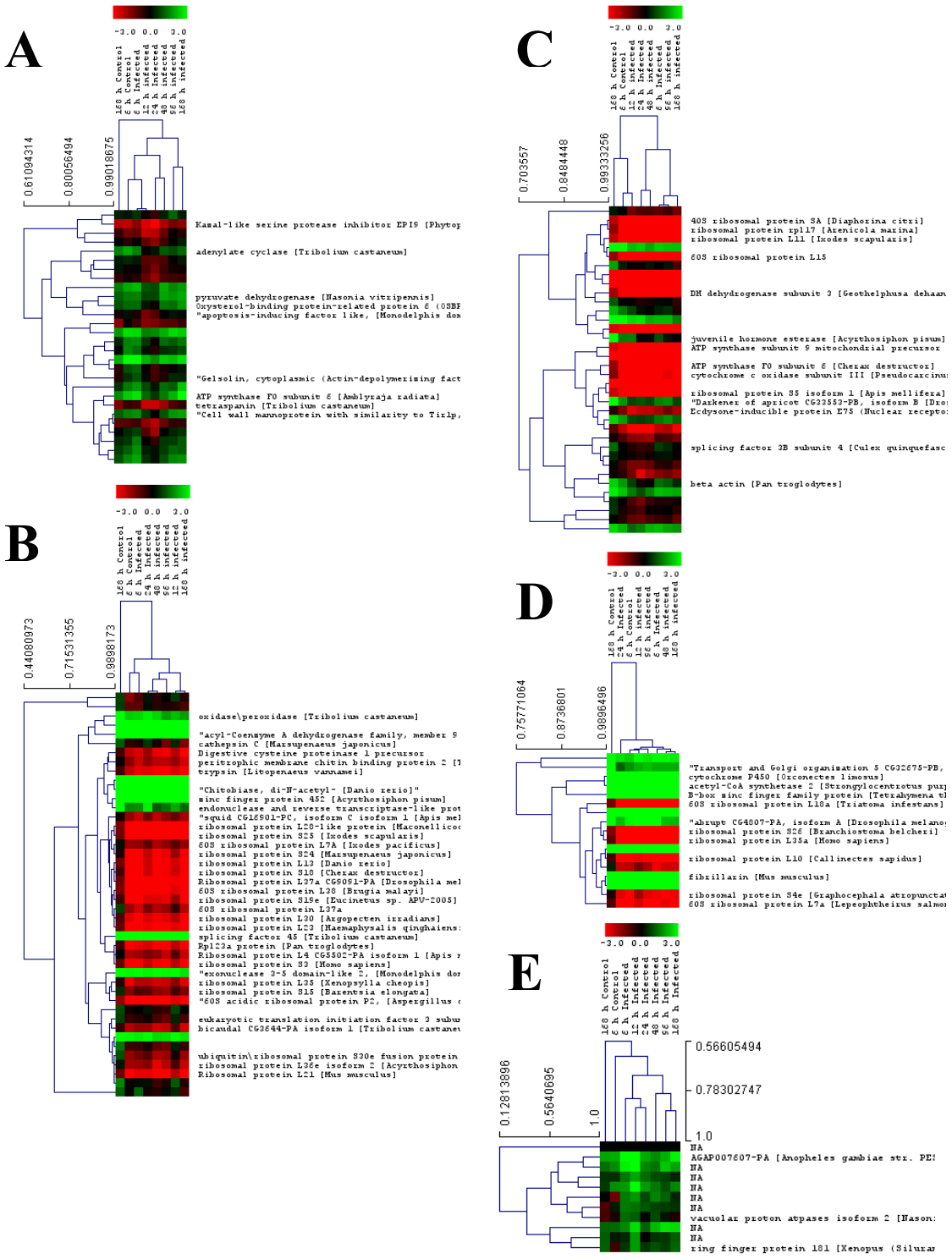

Figure 4.5 Differentially expressed *Homarus americanus* hepatopancreatic genes during WSSV challenge clustered by K-means clustering. Gene expression heat maps illustrate the $\log_2$ ratio of experimental sample/reference sample, where a gradient of red to green represents a threefold or greater decrease in gene expression to a threefold or greater increase in gene expression. (A) cluster 1, (B) cluster 2, (C) cluster 3, (D) cluster 4, (E) cluster 5.

Microarray and RT-qPCR data agree that eTIF3-5e, cathepsin C2, ecdysone-inducible protein E75, and trypsin 1a are differentially expressed based on one-way ANOVA at $\alpha = 0.005$ and $\alpha = 0.05$ respectively. These two gene expression analysis techniques also agree that cathepsin C3, ALFHa-2 and SAA are not differentially expressed. Cathepsin C1, thioredoxin, trypsin 1b, Hsp 21, PEPCK, ALFHa-1 and ALFHa-4 expression is not significantly different with microarray analysis but is significantly different with RT-qPCR analysis (Table 4.2)

The greatest difference in eTIF3-5e expression is seen at the end of the trial when 168 h moribund WSSV infected lobsters have 5.93-fold lower eTIF3-5e expression than their time-matched controls. Cathepsin C1 expression rises in the control lobsters where the expression at the end of the trial is 8.50-fold higher at 168 h. Gene expression of cathepsin C1 in the moribund infected lobsters is decreased 12.58-fold compared to its time-point control. Cathepsin C2 expression drops after 24 h in infected lobsters where 96 h and 168 h infected lobsters have statistically significantly different expression than 24 h infected. The greatest difference in cathepsin 2 expression exists between 168 h infected lobsters and its time-point control where expression dropped 7.62-fold. EIPE75 expression decreases during infection where the expression in 168 h and 96 h infected lobsters is 3.82-fold and 3.14-fold lower than the 168 h control lobsters respectively. Thioredoxin expression rose in infected and control animals where late time points were significantly higher than earlier time-points. Although thioredoxin expression peaked at 5.26-fold the 6 h control animals, it was not statistically different that the 168 h control which was 3.06-fold higher than the 6 h control. Trypsin 1a expression drops after 24 h

Table 4.2 RT-qPCR verification of microarray analysis with expression ratios listed as given time-point/6 h control, except where 168 h infected/ 168h control expression ratio is specified

Gene	Accession #	Measurement Technique	Infected						Control	168 h Infected/ 168 h Control	p-value
			6 h	12 h	24 h	48 h	96 h	168 h	168 h		
Eukaryotic Translation Initiation Factor 3-5e	EH035483	RT-qPCR	0.92	0.78	1.07	0.71	0.49	0.38	2.27	0.17	1.16×10^{-4}
		Microarray	1.07	1.72	1.20	0.89	0.77	0.87	2.11	0.41	0.002
Cathepsin C1	EH116404	RT-qPCR	0.81	0.68	0.89	0.64	0.54	0.68	8.50	0.08	1.29×10^{-4}
		Microarray	1.11	1.72	1.92	1.15	0.71	-0.34	2.49	-0.14	0.008
Cathepsin C2	EV782128	RT-qPCR	0.92	0.88	1.21	0.50	0.33	0.30	2.32	0.13	1.64×10^{-4}
		Microarray	1.00	1.22	1.16	0.94	0.95	0.93	1.41	0.66	0.004
Cathepsin C3	EH116090	RT-qPCR	0.83	0.81	0.67	0.69	0.84	0.47	0.83	0.57	0.249
		Microarray	1.32	1.99	2.11	1.40	1.15	2.52	133.02	0.02	0.3
Ecdysone-inducible protein E75	EX471223	RT-qPCR	0.81	0.60	0.69	0.60	0.45	0.37	1.40	0.26	2.48E-02
		Microarray	0.90	0.81	0.86	0.86	1.03	0.91	1.33	0.68	0.004
Thioredoxin	FD699182	RT-qPCR	1.09	1.13	1.08	1.45	5.26	2.73	3.06	0.89	3.97×10^{-7}
		Microarray	1.01	1.12	1.03	1.08	1.24	1.15	1.10	1.04	0.022
Trypsin 1a	EV781656	RT-qPCR	0.99	0.89	1.10	0.72	0.32	0.25	3.69	0.07	4.66×10^{-5}
		Microarray	0.93	0.96	0.94	0.92	0.88	0.86	1.36	0.63	0.001
Trypsin 1b	EF095144	RT-qPCR	0.80	2.42	0.79	0.50	23.04	3.64	2.06	1.77	2.09×10^{-3}
		Microarray	1.02	1.15	1.05	1.08	1.12	1.09	1.08	1.01	0.472
Hsp 21	DV774085	RT-qPCR	0.82	1.10	0.89	0.82	0.67	0.67	1.94	0.35	2.72×10^{-3}
		Microarray	1.31	8.23	5.09	3.05	2.70	3.45	1.67	2.06	0.006
Phosphoenolpyruvate carboxykinase	DV773064	RT-qPCR	1.16	1.80	0.75	0.49	5.09	1.52	1.71	0.89	2.49×10^{-2}
		Microarray	1.19	1.99	1.41	1.27	1.47	1.80	1.76	1.02	0.025
ALFHa-1	EU625516	RT-qPCR	0.95	1.74	0.52	0.91	2.11	1.42	2.41	0.59	3.48×10^{-2}
		Microarray	1.02	1.15	1.08	1.07	1.06	1.04	1.05	0.99	0.186
ALFHa-2	ALFHa-2	RT-qPCR	1.64	2.35	1.40	1.25	0.93	0.35	1.87	0.18	2.49E-01
		Microarray	1.03	1.13	1.02	1.02	1.01	1.00	1.04	0.95	0.211
ALFHa-4	DV772634	RT-qPCR	0.84	1.24	0.61	1.38	5.40	1.23	2.15	0.57	2.52×10^{-4}
		Microarray	1.08	1.43	1.14	1.35	1.36	1.10	1.19	0.93	0.076
SAA	EH116055	RT-qPCR	0.27	0.50	0.50	1.28	3.93	1.15	0.73	1.57	0.180
		Microarray	1.00	1.31	1.10	1.38	1.22	1.05	1.04	1.01	0.123

where 6 h, 12 h and 24 h infected samples are higher than 168 h infected animals. The largest difference in expression for Trypsin 1a is for 96 h and 168 h infected lobsters where there is an 11.43–fold and 14.95–fold decrease in expression compared to the 168 h control. Trypsin 1b expression peaks at 96 h in the infected animals. The expression of trypsin 1b is 11.19-fold higher in the 96 h infected samples compared to the 168 h control animals. Heat shock protein 21 decreased in infected samples from 24 h to 168 h. The Hsp 21 expression of 168 h infected treatment animals is downregulated 2.89-fold compared to its time-point control. PEPCK expression peaked at 96 h in infected animals but was only statistically significantly different than the 24 h and 48 h infected samples at 6.83-fold and 10.42-fold higher, and was not statistically different than the control lobsters. ALFHa-1 expression was only moderately different between treatments where the 96 h infected and 168 h control treatment lobsters were 4.10-fold and 4.69-fold higher than the 24 h infected treatment. ALFHa-4 expression on the other hand peaked at 96 h in the infected samples and was higher than all other infected samples and the 6 h control. The difference in ALFHa-1 expression was greatest between the 96 h and 24 h infected treatments with 96 h treatment expression 8.80-fold higher.

4.5 Discussion

This is the first study to use a high-throughput microarray technique to analyze the response of the American lobster (*H. americanus*) to a viral challenge. The goal of this project was to begin to characterize the genes involved in mediating the response of *H. americanus* to a viral pathogen. There is currently no known naturally occurring viral pathogen so *H. americanus* was challenged with WSSV as it is known to infect a wide

variety of decapod crustaceans (Sánchez-Paz, 2010). The *in vivo* WSSV injection challenge was also conducted to determine if *H. americanus* is susceptible to WSSV, and could therefore become a valuable model of viral disease. White Spot Syndrome Virus infected lobsters exhibited a decrease in THC that was statistically significant by the time they became moribund at 168 h (Fig. 4.1). Earlier pilot studies found that lobsters challenged with WSSV died within two weeks after a drop in THC (personal communication with Dr. Philip Byrne). It was critical to lethally sample lobsters before they died in this study because RNA rapidly degrades and becomes unusable in a deceased lobster. Routine histology found classical signs of WSSV infection in the antennal gland of WSSV infected lobsters after 168 h and this was confirmed by electron microscopy. The presence and replication of WSSV was also confirmed with qPCR on lobster haemolymph, which found increasing amounts of WSSV in infected lobsters as the trial progressed. Taken together, the clinical, histological and molecular findings demonstrate that WSSV is able to infect and replicate in *H. americanus* tissues of lobsters held at 20 ^{o}C.

This study used 3% NaCl instead of WSSV-free shrimp homogenate as a control due to the unavailability of specific pathogen-free shrimp. A high proportions of available commodity shrimp has been shown to contain WSSV (personal observation) and the short duration of the funding limited the possibility of obtaining WSSV free shrimp even after several months and multiple suppliers. However, this does not significantly impact the results of this study as the impact of an exposure to a limited amount of shrimp

homogenate early in the trial is quickly outweighed by the significant impact of a rapidly replicating WSSV.

The WSSV infection challenge provided a viral model of disease in *H. americanus* from which to determine the lobster's response to a viral pathogen. Lobster hepatopancreatic tissue was used to determine the molecular immune response because the hepatopancreas is the major site of humoral immune factors (Factor and Beekman, 1990). Microarray transcriptional analysis provides an excellent method for determining molecular mediators of the lobster's immune response and has previously been used during *A. viridans* and *A. haemophila* challenges (Chapter 2 and 3). One-way ANOVA analysis with 1000 permutations at $\alpha = 0.005$, was chosen as the statistical cut-off for biological meaningful differences in gene expression. Overly harsh correction of gene expression data with Bonferroni correction leaves us with an unacceptably high number for false negatives. This statistical cut-off has proven the most biologically relevant in our previous studies of lobster response to pathogens (Chapter 2 and 3). Sample HC of the 136 differentially expressed genes at $\alpha = 0.005$ is better at separating immune genes from control genes, then when Bonferroni correction is applied at $\alpha = 0.05$, and therefore contains more genes primarily responsive to WSSV infection. By reducing the statistical stringency by omitting false-discovery correction, we have increased the number of false-positives, but this is appropriate because our findings are verified with the more sensitive gene expression analysis technique of RT-qPCR. We are at a very early stage in discovering *H. americanus* immune genes; therefore it is better to use biological information in conjunction with statistical analysis on a large suite of genes containing

some potential false-positives whose expression will be verified by complementary approaches. Restricting potential immune mediators with harsh statistical correction will result in missing novel genes involved in pathogen response.

Only a subset of the 136 genes that are differentially expressed in response to WSSV will be genes primarily involved in the lobster immune system. We obtained functional information by comparing differential expressed lobster gene sequences with protein sequences in GenBank, to help identify which genes may be involved in lobster immunity. Less than 54% of these genes were similar to proteins in GenBank and therefore many of the genes identified by microarray have no corresponding functional information. Genes were grouped based on the similarity of their average treatment expression over the course of the viral challenge to provide some additional information on gene function. A K-means cluster analysis grouped all the genes into five expression patterns (Fig. 4.5 and Appendix C Table C.1). Treatment HC of these five expression patterns begins to elucidate a functional role for genes within a single cluster. Gene expression is not independent, and therefore genes with similar expression profiles are more likely to be under similar transcriptional controls, and have similar physiological function (Eisen et al., 1998).

Cluster 1 sample HC indicates that genes in this cluster are not involved in early response to infection as seen by the similarity of 6 h and 12 h infected treatments with the 6 h control. The similarity between infected time-points, but not 168 h control, indicates that these genes are likely involved in immune response as infection progresses because late

infection time-points are more similar to each other than they are to early infection time-points. Cluster 1 contains genes where 32.1% had similarity to proteins in GenBank. Two of the genes in cluster 1 are likely immune candidates: kasal-like serine protease inhibitor (FE535180) and apoptosis-inducing factor-like protein (FE043558). Genes with similar expression patterns to the aforementioned genes are likely also involved in lobster immune response. Cluster 5 sample HC indicates that these genes are also involved in lobster response to WSSV infection as the control and infected treatments are more similar within their treatment class than between them. There is more similarity in late infection treatments than earlier infected time-points and therefore response is based on progression of WSSV infection and not WSSV infection in general. Only 27.3 % of the genes in cluster five have similarity with genes in GenBank.

RT-qPCR based verification of microarray gene expression was performed on 14 genes (Table 4.2). Four of these genes, eTIF3-5e, cathepsin C2, EIPE75 and trypsin 1a, were chosen to verify that genes found to be differentially expressed at $\alpha = 0.005$ on the microarray were truly differentially expressed. The other genes were chosen because they are functionally similar to these four significant genes, or they have been previously implicated in *H. americanus* or crustacean immunity.

eTIF3-5e is differentially expressed during WSSV infection, where expression inversely correlates with infection time. Another eukaryotic translation initiation factor, eTIF5A, is reported to have a role in slowing WSSV infection, where its expression decreases as infected shrimp become moribund (Phongdara et al., 2007). The functional role of eTIF3-

5e is not known and why its expression decreases in WSSV lobsters is unknown at this time.

Cathepsin C, also known as dipeptidyl peptidase I, is a lysosomal cysteine protease (McDonald et al., 1972; Kominami et al., 1992). Cathepsin C is capable of activating many chymotrypsin-like serine proteases and has been found to be a central coordinator for the activation of many serine proteinases in immune and inflammatory cells (McEuen et al., 1998; Smyth et al., 1995).

Cathepsin C is also reported to be critically involved in cytotoxic metabolite generation and a regulator of immune cell functions (Thiele et al., 1997). Wang et al. (2012) has also shown that cathepsin C plays a crucial role in antiviral response to WSSV in the Chinese shrimp *Fenneropenaeus chinensis*, where hepatopancreatic expression is upregulated almost 6-fold after 24 h. Cathepsin C also has a role in antibacterial immunity where it is upregulated after *Vibrio anguillarum* in *F. chinensis* (Wang et al., 2012) and Chinese mitten crab *Eriocheir sinensis* (Li et al., 2010) and in response to LPS in the black tiger shrimp *Penaeus monodon* (Qiu et al., 2009). Microarray analysis revealed that one cathepsin C isoform, C2, was significantly differentially regulated but two other isoforms, C1 and C3, were not. RT-qPCR analysis was performed on all three isoforms to determine if there was isoform specific response to WSSV. RT-qPCR found that both cathepsin C1 and C2 are differentially expressed but cathepsin C3 is not. Both cathepsin C1 and C2 expression decreased during the course of WSSV infection until it was downregulated 12.58-fold and 7.62-fold in moribund lobsters compared to their time-

point control. This decrease in expression is the opposite of what is seen in shrimp anti-WSSV and antibacterial response (Qiu et al., 2009; Wang et al., 2012) however, cathepsin expression was only measured for the first 24 h in those studies. Li et al. (2010) measured cathepsin expression in *E. sinensis* haemocytes until 48 h and found that after an initial peak at 6 h, expression decreased to control levels by the end of their experiment at 48 h. Another difference between this study and the shrimp and crab studies is that they used β-actin expression as their sole reference gene and our findings indicate that β-actin was differentially expressed during WSSV infection in *H. americanus*.

EIPE75 has a critical role in regulation of the moulting process of arthropods (Mane-Padros et al., 2008). During WSSV infection, EIPE75 decreased to 3.82-fold less in moribund infected lobsters than its time-point control, indicating an inhibition of the moulting process. These findings match the expression of *L. vannamei* ecdysone related gene during WSSV infection (Zhao et al., 2007).

Thioredoxin is a redox protein that aids in the neutralization of reactive oxygen species (ROS). Thioredoxin was implicated in the immune response of *H. americanus* to *A. viridans* where it was significantly upregulated in moribund animals, but not in *A. haemophila* defence. Thioredoxin expression increases during WSSV infection and expression in 96 h infected animals is higher than any infected or control time-point before it; however, it is not statistically different than the 168 h control. This indicates that the increase in thioredoxin expression could be due to the time in the trial and not necessarily due to WSSV infection. The lobsters in this trial were held at 20 ^{o}C which

may have caused stress despite the slow acclimation period before the trial began. Thioredoxin may prove to be a valuable biomarker for thermal stress as well as ROS release.

Trypsin 1a and 1b are serine proteases, a class of proteases known to have significant roles mediating crustacean immunity by regulating protein signaling cascades (Cerenius and Söderhäll, 2004). Shi et al. (2009) has identified four trypsin-like genes that are involved in the Chinese shrimp (*F. chinensis*) response to WSSV infection, where all four trypsin isoforms are upregulated in hepatopancreatic tissue. *H. americanus* trypsin 1a expression decreases during WSSV infection where the 168 h moribund infected lobsters expressed 14.95-fold less trypsin 1a than the 168 h control. Conversely, trypsin 1b expression rose during the trial to peak at 11.19-fold and 23.04-fold higher expression in the 96 h infected lobsters compared to the 168 h and 6 h controls. The difference between the trypsin isoforms highlights an isoform specific reaction to WSSV infection. *H. americanus* trypsin 1a and 1b expression during infection with the parasitic scuticociliate *A. haemophila* revealed that trypsin 1a is moderately increased early in the infection while trypsin 1b expression increased over 10-fold in the moribund infected lobsters compared to its time-point control (Chapter 3). Trypsin 1b has also been shown to be up regulated almost 70-fold during *H. americanus* infection with the Gram-positive bacterium *A. viridans* while trypsin 1a expression remained unchanged (Chapter 3). The viral, bacterial and parasitic studies of *H. americanus* immune response have highlighted the differential expression of the trypsin 1b isoform, over trypsin 1a, and its role in *H. americanus* immune response at late stages of pathogen infection.

Hsp 21 belongs to a family of heat shock proteins that are expressed to promote cell survival by refolding proteins and preventing denaturation during stressful events (Feder and Hofmann, 1999). They also participate in normal cellular processes such as protein trafficking DNA replication, protein synthesis and signal transduction (Hartl, 1996). Our findings indicate that Hsp 21 expression is down 2.89-fold at 168 h compared to its time-point control. This down regulation was surprising as Hsp 21 expression was expected to rise during WSSV infection. However, Hsp21 was also found to be downregulated 10-fold, 72h after WSSV infection in *P. monodon* (Huang et al., 2008). Hsp27 has a functional role in apoptosis, and repression of small Hsp expression could lead to increased viral replication within a cell (Mehlen et al., 1996). Apoptosis is considered an important cellular defence mechanism to WSSV in multiple tissues (Wongprasert et al., 2003). Inhibition of apoptosis would allow WSSV to proliferate and this could explain the decrease that was seen in potentially proapoptotic Hsp 21 (Leu et al., 2013).

PEPCK is the rate limiting step in gluconeogenesis. Shrimp plasma glucose and lactate concentrations during WSSV infection become depleted by over 65% at 36 h, plasma triglyceride concentration decrease by over 75% at 72 h (Chen et al., 2011) and the TCA cycle slows down by 72 h post-WSSV infection (Mohankumar and Ramasamy, 2006). Chen et al. (2011) found glucose-6-phosphate dehydrogenase expression increases by 72 h, thereby shunting glucose-6-phosphate into the pentose phosphate pathway (PPP). The PPP generates reducing reagents capable of combating ROS and making the sugar backbone required for the synthesis of nucleotides that WSSV requires for replication.

Therefore, the increase in products from the PPP could simultaneously protect WSSV from ROS generated from the *H. americanus* immune system while facilitating WSSV replication. PEPCK increases during infection and was statistically significantly higher at 96 h compared to 24 h or 48 h infected animals. The increase in PEPCK expression could be in response to the decrease in plasma glucose, where increased PEPCK expression could either drive gluconeogenesis, or generate substrate for glucose-6-phosphate dehydrogenase. Additional study will be required to look at the gene expression of enzymes along the glycolysis/gluconeogenesis pathway.

Anti-lipopolysaccharide factors are antimicrobial peptides that can inhibit or kill pathogenic microorganisms (Aketagawa et al., 1986; Hauton, 2012). Increased expression of *H. americanus* ALF isoforms has previously been shown during Gram-negative and Gram-positive bacterial infections (Beale et al., 2008; Chapter 2), as well as during infection with the parasitic scuticociliate *A. haemophila* (Chapter 3). All of these studies noted that there was differential expression of the ALFHa isoforms where *A. haemophila* infection caused the greatest increases in ALFHa-2 expression while *A. viridans* infection caused the greatest increases in ALFHa-4 expression (Chapter 2 and 3). Studies of shrimp and crayfish immune response to WSSV have also demonstrated the important role that ALF have in inhibiting WSSV infection (Leu et al., 2013). None of the 6 ALFHa isoforms that were measured on our microarray (ALFHa-1, ALFHa-2, ALFHa-3, ALFHa-4, ALFHa-6 and ALFHa-7) were found to be differentially expressed.

RT-qPCR was used to confirm the microarray findings of no differential expression of ALFHa-1, ALFHa-2 and ALFHa-4 isoforms because of its increased sensitive and accuracy. ALFHa-1 and ALFHa-4 were found to be differentially expressed during infection, peaking at over 4-fold and 8-fold the 24 h infected animals at 96 h post infection respectively. However, the expression of ALFHa-1, ALFHa-2 and ALFHa-4 was not different between the 168 h moribund infected animals and their time-point control. This finding indicates that ALFHa isoforms do not play a role in mediating immunity to WSSV, and perhaps to viral infections in general. This is in stark contrast to the large ALFHa isoform gene expression increases during bacterial and parasitic infection (Chapter 2 and 3) and suggests specificity for these types of pathogens.

SAA is an acute phase protein routinely used in human and veterinary medicine as a widely recognised indicator of innate immune activation in vertebrates (Cray et al., 2009). Previous studies have demonstrated that increases in SAA expression correlate with progressively severe infections of both of *A. viridans* and *A. haemophila* in *H. americanus* (Chapter 2 and 3). However, there was no differential expression of SAA found through either microarray or RT-qPCR analysis. This indicates that while SAA is a good indicator of compromised health in *H. americanus* due to bacterial or parasitic infections, it does not hold true for WSSV infections. Perhaps SAA is not differentially expressed because WSSV is capable of circumventing the *H. americanus* immune system or perhaps it is a function of temperature as the *in vivo A. viridans* and *A. haemophila* challenges were conducted at 15 ^{o}C and 2 ^{o}C respectively, while this WSSV challenge was conducted at 20 ^{o}C.

The clinical, histological and molecular findings from our WSSV injection challenge suggest that WSSV is able to infect and replicate in *H. americanus* tissues of lobsters held at 20 ^{o}C. Whether WSSV is able to infect, persist or replicate in *H. americanus* below 20 ^{o}C remains to be seen. Either way, we have established the first viral model of disease for *H. americanus*. Comparison of the differentially expressed genes found during the microarray analysis with RT-qPCR has demonstrated that the *H. americanus* microarray is good at detecting differentially expressed genes. However, the microarray is not as good at predicting all of the differentially expressed genes as several false-negatives were found through RT-qPCR. This finding indicates that the microarray is good at finding true-positives at the expense of false-negatives for differentially expressed genes; thereby providing additional evidence for the case of reduced statistical stringency combined with biologically meaningful functional information to find molecular mediators of *H. americanus* immunity.

ALFHa isoforms and SAA expression is not involved in *H. americanus* immune response to viral infection in stark contrast to bacterial and parasitic infection (Chapter 2 and 3). This represents an interesting pathogen class-specific response by *H. americanus* and demonstrates the ability of the *H. americanus* innate immune system to respond differently to specific classes of pathogens. Another interesting finding is the increased expression of trypsin 1b in the late stages of infection to bacterial, parasitic and viral pathogens (Chapter 2 and 3). The expression of this trypsin-like serine protease should be

examined in more detail to help elucidate how it is involved in the *H. americanus* immune system.

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Supplementary Data associated with chapter 4 can be found in Appendix C.

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Chapter 5: *H. americanus* Molecular Immune Molecule Discovery: Summary, Discussion and Conclusions

5.1 General Considerations

American lobster *in vivo* pathogen challenges can help us discover novel primary lobster immune molecules that can be extrapolated and compared to crustacean immune response in general. These pathogen challenges can also help us discover the plethora of secondary immune responses, such as metabolic changes that are also critical to immune response. They can also help us discover biomarkers of disease, stress and overall health status in lobsters, which are essential because there is currently no definitive clinical indicators of lobster health. These clinical indicators of lobster health hold commercial utility as the implementation of programs to screen lobsters for health prior to entry into the secure live-storage facility, could mitigate mass-mortality events which lead to large economic loss. Additionally, lobster health biomarkers would be valuable tools for examining epidemiological occurrence of pathogen outbreaks in the wild or in clinical cases in live-lobster holding facilities, or understanding of the impacts of anthropogenic stressors on lobster immune response.

By comparing and contrasting *in vivo* challenges from different classes of pathogens, one can quickly determine if lobsters respond with a broad and non-specific immune response or whether specificity exists to different pathogen classes. *A. viridans* and *A. haemophila* are respectively major warm water (>10 ^{o}C) and cold water (<5 ^{o}C) causes of lobster mortality and morbidity. Rudimentary aspects of lobster immunity has been examined in relation to these pathogens, but only from clinical, histological, gross anatomic and basic

humoral characterization (Cornick and Stewart 1968; 1973; 1978; Acton et al., 1969; Stewart and Zwicker, 1972; Mori and Stewart, 1978; Goldenberg et al., 1984; Chisholm and Smith, 1995; Anderson and Beaven, 2005; DeGuise et al., 2005; Christie et al., 2007; Battison et al., 2008; Battison and Summerfield, 2009). This is satisfactory for examining cellular immunity, but it drastically underestimates the immune response by missing the intricacies and nuances of the humoral molecular immune response. The hepatopancreas is the primary organ responsible for humoral immunity in *H. americanus* (Factor and Beekman, 1990) and crustaceans in general (Shields et al., 2006). This is why the hepatopancreas was chosen as the tissue from which transcriptomic changes would be monitored using a high-throughput microarray.

5.2 *H. americanus* Molecular Immune Response to a Bacterial Challenge

The first objective of the study in chapter 2 was to verify that the *A. viridans* var. *homari in vivo* pathogen challenge was causing infection, and that lobsters were mounting an immune response. Clinical and histological examination confirmed that fixed and circulating haemocytes were phagocytosing Gram-positive tetrad-cocci, characteristic of *A. viridans*, and that there was bacterial proliferation, both in the fixed phagocytes of the hepatopancreas and in the circulating haemolymph. The progression of infection was concomitant with marked haemocytopenia, a typical clinical finding in gaffkemia (Rabin 1965; Rabin and Hughes, 1968; Cornick and Stewart, 1968; Stewart et al., 1968; 1969a; 1969b; Stewart and Zwicker, 1972).

Transcriptomic analysis of gene expression via microarray discovered 148 genes that were differentially expressed during *A. viridans* infection. A number of these genes share similarity with proteins that belong to families of traditional immune molecules including: ALFHa-1, ALFHa-2, ALFHa-4 and ALFHa-6, SAA, thioredoxin 1, haemocyanin subunit 3 and a serine protease inhibitor.

The differential gene expression found using the lobster microarray was verified using RT-qPCR. Verification was performed on all of the aforementioned genes, with the exception of ALFHa-6, along with a differentially expressed gene with similarity to hexokinase. Verification was also performed on two trypsin isoforms, where trypsin 1b was differentially expressed and trypsin 1a was not. RT-qPCR confirmed the differential expression of all of these genes in infected lobsters compared to controls, with the exception of trypsin 1a.

The most significantly differentially expressed genes can be found by comparing the moribund infected lobsters, 78 h infected, to their time-point controls. Trypsin 1b and SAA are expressed over 54-fold and 30-fold higher in moribund infected lobsters while ALFHa-1, ALFHa-2 and ALFHa-4 are expressed over 12-fold, 13-fold and 26-fold higher, respectively. The increase in expression for SAA is significant because it has long been used as an indicator of innate immune activation in human and veterinary medicine but it has not been studied in crustacean immunology. The over 30-fold increase in expression during *A. viridans* infections distinguishes it as a potentially very valuable biomarker of bacterial infection. The largest change in gene expression is found for a

gene that is not traditionally known as an immune factor, and that is trypsin 1b. The over 54-fold increase in expression, while the trypsin 1a isoform is unchanged, is exceptionally interesting. Trypsin is a serine protease and serine proteases are known to be critical mediators of immune cascades in crustaceans during processes such as prophenoloxidase mediated melanisation and haemolymph clotting (Cerenius and Söderhäll, 2004). This trypsin isoform could have a dual role in metabolism and immunity, or perhaps future bioinformatic analysis combined with the sequencing of the entire trypsin 1b gene will show that it is more similar to immunological serine proteases than metabolic trypsins. The microarray that was used to measure gene expression in this study measures the expression of six ALFHa isoforms. Four of the six ALFHa isoforms were differentially expressed and this was verified for the three RT-qPCR tested isoforms: ALFHa-1, ALFHa-2 and ALFHa-4. ALFHa-3 and ALFHa-7 isoforms were not differentially expressed. This suggests that there is isoform-specific expression of the ALFHa family and not a broad "all-or-nothing" immune response. Perhaps the expression of ALFHa-3 and ALFHa-7 are important to another class of pathogens.

5.3 *H. americanus* Molecular Immune Response to a Eukaryotic Parasitic Challenge

Chapter three focused on the immune response of lobsters to the cold water (<5 ^{o}C) eukaryotic parasite *A. haemophila* that causes bumper car disease, a lethal disease that usually takes 4-14 weeks to kill its host. Clinical and histological analysis revealed that *A. haemophila* was found in the greatest numbers in the gills, heart and connective tissue surrounding the testis for the first five weeks following the pathogen challenge. This preference for gill, heart and testicular connective tissue continued throughout the infection. During the first four weeks of infection there were no changes in the

concentrations of circulating haemocytes between infected and control animals, but a decreasing trend began at week five in the infected lobsters, while the control lobsters remained relatively steady (Fig 3.1). There was also a marked rise in the concentration of circulating haemocytes within the first 48 h for both infected and control lobsters, but this decreased and remained steady for four weeks in the infected animals, and for the remainder of the experiment in the control lobsters. Histological examination during the first five weeks found many haemocyte aggregations surrounding individual *A. haemophila* parasites in infected lobsters, and no haemocyte aggregations in control lobsters. After five weeks the number of visible haemocyte aggregations decreased dramatically in infected lobsters and this corresponded directly with a decrease in circulating haemocytes, and a proliferation of *A. haemophila*. These findings are typical of *A. haemophila* infections (Cawthorn, 1997; Athanassopoulou et al., 2004) and demonstrate that the lobster immune system is trying to combat the infection.

Transcriptomic analysis of the hepatopancreas via microarray analysis revealed 145 differentially expressed genes. Several of these 145 genes have similarity to proteins that have been recognised as traditional immune factors including: a serine protease inhibitor, three haemocyanin subunits, vitelline membrane outer layer protein, two pseudohaemocyanin-2 precursors, a C-type lectin receptor, heat shock protein 70, thioredoxin 2, a Toll-like receptor, ALFHa-4, ALFHa-6 and SAA. Several of these genes were verified by RT-qPCR including SAA, ALFHa-4, C-type lectin receptor, and pseudohaemocyanin-2 precursor. In addition, ALFHa-1 and ALFHa-2 were verified due to their sequence similarity to ALFHa-4 and ALFHa-6, and their involvement in *A.*

viridans immune response (Chapter 2). Trypsin 1a and Trypsin 1b and thioredoxin 1 were also included because of their involvement in *A. viridans* immunity (Chapter 2). RT-qPCR determined that there was over 54-fold, 35-fold 10-fold, 8-fold and 6-fold increases in gene expression of SAA, ALFHa-2, trypsin 1b, ALFHa-4 and ALFHa-1 in moribund infected lobsters over their time point controls. When the 8 week infected animals where compared to their time point control the expression of SAA, ALFHa-2, ALFHa-4, ALFHa-1 and trypsin 1b is increased over 19-fold, 17-fold, 13-fold, 9-fold and 12-fold respectively. All of these genes were also increased in response to *A. viridans* infection (Chapter 2) and are again involved in lobster immune response to *A. haemophila* (Chapter 3). This is compelling evidence that ALFHa isoforms, SAA and trypsin 1b are involved in the *H. americanus* primary immune response and that they increase during disease progression.

C-type lectin receptor may be involved in lobster immunity but this may be restricted to the early stages of infection as it peaks at 24 h and then drops throughout the trial. Trypsin 1a expression is similar to pseudohaemocyanin-2 precursor expression in that their expression steadily decreases as the trial progresses, and both are not significantly different than their respective time point controls. This expression decrease may be a function of metabolism over the duration of the trial, and reflective of live-lobster holding in the absence of feeding. Thioredoxin 1 was not differentially expressed via microarray and this was verified with RT-qPCR. Despite its increase during *A. viridans* infection (Chapter 2), it is not differentially expressed during *A. haemophila* infection (Chapter 3).

5.4 *H. americanus* Molecular Immune Response to a Viral Challenge

There is no known viral pathogen of *H. americanus* so a viral disease model had to be generated prior to our immune response analysis. White Spot Syndrome Virus is a devastating viral disease of farmed shrimp, *L. vannamei* and *P. monodon*, that has been proposed by the European Union and OIE to be capable of infecting all decapod crustaceans (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:en:PDF; http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.2.06_WSD.pdf). Regulatory investigations into the potential susceptibility of *H. americanus* to WSSV were being carried out by Dr. Phil Byrne at the Charlottetown Aquatic Animal Pathogen Biocontainment Laboratory which provided an excellent opportunity to collaborate and develop a WSSV infection model of *H. americanus*, if it was susceptible.

Clinical and histological analysis found that circulating haemocytes decrease in lobster which had been injected with WSSV compared to control animals. Light and electron microscopy also revealed that WSSV is present and replicating in antennal gland tissue. An additional diagnostic test was performed using qPCR, detecting vp28 of WSSV, and found that the concentration of WSSV increased in the haemolymph of WSSV infected lobsters over the length of the trial (Chapter 4).

This clinical, histological and molecular evidence of WSSV replication verified that a model of viral infection in *H. americanus* has been generated. This model was used to measure the transcriptomic changes that occurred in the hepatopancreas as the infection

progressed. Microarray analysis found 136 differentially expressed genes, only one of which was similar to a protein in GenBank that has a traditional immunological function: Kazal-like serine protease inhibitor.

Several genes were chosen to verify the microarray gene expression findings including four that were differentially expressed at $\alpha = 0.005$ (eTIF3-5e, cathepsin C2, EIPE75 and trypsin 1a). Eight other genes were verified because they either had similarity to these significant genes, or they had been previously implicated in crustacean immunity including: cathepsin C1, cathepsin C3, thioredoxin, trypsin 1b, Hsp 21, PEPCK, ALFHa-1, ALFHa-2, ALFHa-4 and SAA. Cathepsin C expression can play a role in activating serine proteases cascades in crustacean immunity (Qiu et al., 2009; Li et al., 2010; Wang et al., 2012). Cathepsin C2 and cathepsin C1 expression decreased during WSSV infection to the point where they were over 7-fold and 12-fold lower in infected lobsters at the end of the trial than control lobsters. The expression of cathepsin C3 is unchanged between infected and control lobsters throughout the experiment. The isoform specific expression of cathepsin C family members raises an interesting question of which pathways they may be responsible for activating, and what kind of substrate specificity or overlap may exist.

Trypsin 1a expression decreased throughout the experiment until the infected lobsters expressed over 14-fold less trypsin 1a at the end of the trial than the control lobsters. Contrary to this, trypsin 1b expression peaked at 96h in infected lobsters where it was over 23-fold and 11-fold higher in infected lobsters than the control lobsters at the

beginning and end of the experiment respectively. This again points to isoform specific expression where trypsin 1b expression increased in infected lobsters whether it is a bacterial infection (Chapter 2), a parasitic infection (Chapter 3) or a viral infection (Chapter 4).

The expression of thioredoxin is difficult to interpret as it is increased over 5-fold in infected lobsters at 96 h compared to the 6 h control, but this increase in expression is not significantly different than the 168 h control. The 168 h control lobsters are significantly higher than at all time points before 96 h for both infected and control animals. This leads to the possibility that thioredoxin expression increases as a function of time in the trial at 20 ^{o}C, and perhaps it could be a marker of temperature stress, and not immune response, in this case.

ALFHa isoforms and SAA have been strongly involved in the *H. americanus* immune response to bacterial (Chapter 2) and parasitic infections (Chapter 3) so it was surprising to see that they were not differentially expressed, as a means of immune response, as determined by either microarray or RT-qPCR. This leads to the possibility that WSSV is either 1) capable of moderating the lobster immune response, 2) unrecognizable to the lobster immune response or 3) perhaps it is a function of temperature where at 20 ^{o}C the lobster's immune response is significantly impaired due to temperature stress.

5.5 Specificity and Scope of *H. americanus* Molecular Immune Response

The goal of this PhD research has been to discover how the humoral immune response of *H. americanus* responds to different classes of pathogens. Transcriptomic analysis can find differentially expressed genes but it is critical to use the appropriate statistical stringency to ensure that differentially expressed immune candidates genes are found. The manufacturing process used to generate spotted oligonucleotide microarrays introduces inherent variability into the assay. Each microarray feature contains the same amount of DNA, but the physiochemical properties of each feature means that it will behave in different ways once it comes into contact with the aminosaline-coated surface of the glass slide. This is why the features of the array could have different shapes: small, large, circular or oblong. In addition, the entire 15,376 feature array is spotted by 16 pins, where each block of 961 features is spotted by a single pin. All of these variations are dealt with through spot feature evaluation, and LOWESS normalization to minimize the differences that may occur due to feature-to-feature variation. Nevertheless, the variation introduced by the microarray manufacturing process alone already increases the difficulty in finding true differentially expressed genes.

The most appropriate cutoff for differential expression in *H. americanus* immune response genes was an $\alpha = 005$ combined with functional biological information (Chapters 2, 3 and 4). If the statistical stringency is increased with Bonferroni correction, only true-positives are left but there is an unacceptably high number of false-negatives. Lobster immunology is at a very early stage of *H. americanus* immune molecule discovery, and crustacean immunology in general, it is much more relevant to limit false-

negatives as false-positive will be discovered in the RT-qPCR verification process. RT-qPCR verification is essential as it is a more specific and more accurate measure of gene expression.

The lobster microarray that has been used underestimates the magnitude of the gene expression changes that occur. As an example, microarray analysis found that the difference in expression between bacteria-infected moribund lobsters, and their time point controls, was 6.94-fold, 6.55-fold and 10.45 fold for trypsin 1b, SAA and ALFHa-4 respectively; while RT-qPCR analysis determined that it was actually 54.37-fold, 30.64-fold and 26.70-fold. In addition, the microarray was more prone to false-negatives than RT-qPCR, where cathepsin C1, EIPE75, thioredoxin, trypsin 1b, Hsp 21, PEPCK, ALFHa-1 and ALFHa-4 were determined not to be differentially expressed during the WSSV challenge trial, when RT-qPCR determined that they actually were differentially expressed either because of WSSV infection or trial duration (Chapter 4).

The bacterial (Chapter 2), parasitic (Chapter 3) and viral (Chapter 4) challenges were all designed to measure the *H. americanus* response to pathogenic pressures. All of these experiments can be compared individually or as a whole. This allows the investigations of whether *H. americanus* mounts a broad immune response to all pathogens and stresses, or if there is specificity in the response. Comparison of the differentially expressed genes, found via microarray, between the different pathogens at a statistical cutoff of $\alpha = 0.005$ found that *A. viridans* and *A. haemophila* share 7 genes in their response, even though there are 148 and 145 differentially expressed genes in total (Table 5.1). This represents

less than 5% of the differentially expressed genes for each pathogen. There are even less pathogen response genes in common when compared to WSSV where one differentially expressed gene is shared between *A. viridans* and WSSV, and none between *A. haemophila* and WSSV. Several of the pathogen responsive genes in common between *A. viridans* and *A. haemophila* have immune functions including ALFHa-4, ALFHa-6, SAA and haemocyanin subunit 3. These genes have never been implicated in *H. americanus* immune response before, and SAA has never been implicated in crustacean immunology in general.

RT-qPCR increased the number of common pathogen responsive genes found to include the differential expression of ALFHa-1 and ALFHa-2 between *A. viridans* and *A. haemophila* and trypsin 1b differential expression between all three pathogens (Fig. 5.1). The common differential expression of only ~6% of the genes differentially expressed to respective pathogen in the bacterial and parasitic challenges, and even less between the viral and bacterial or parasitic challenges, is compelling evidence of at least a pathogen-class specific immune response, and not the broad non-specific immune reaction that crustacean innate immune systems were believed to have. This marks an exciting time in lobster immunology, and crustacean immunology in general, where additional studies will no doubt begin to bring about an immunological paradigm shift that recognizes the ability of the crustacean innate immune system to respond differently, if not specifically, to different pathogenic pressures.

Table 5.1 Differentially expressed *Homarus americanus* genes in common between two or more listed pathogens as determines by microarray or RT-qPCR analysis.

	Aerococcus viridans* and *Anophryoides haemophila		***Aerococcus viridans* and WSSV**		***Anophryoides haemophila* and WSSV**		***Aerococcus viridans*, *Anophryoides haemophila* and WSSV**	
	Gene	Accession #	Gene	Accession #	Gene	Accession #	Gene	Accession #
Microarray	SAA	CN949986			NA	DV771836		
	ALFHa-4	EX568422						
	ALFHa-6	DV774220						
	hemocyanin subunit 3	CN950724						
	reticulocalbin	EY290811						
	segmentation protein cap'n'collar	EH116633						
	NA	CN950902						
RT-qPCR	Trypsin 1b	EF095144	Trypsin 1b	EF095144	Trypsin 1b	EF095144	Trypsin 1b	EF095144
	ALFHa-1	FF277612						
	ALFHa-2	FD483938						

NA refers to an EST with no similarity to any protein in GenBank

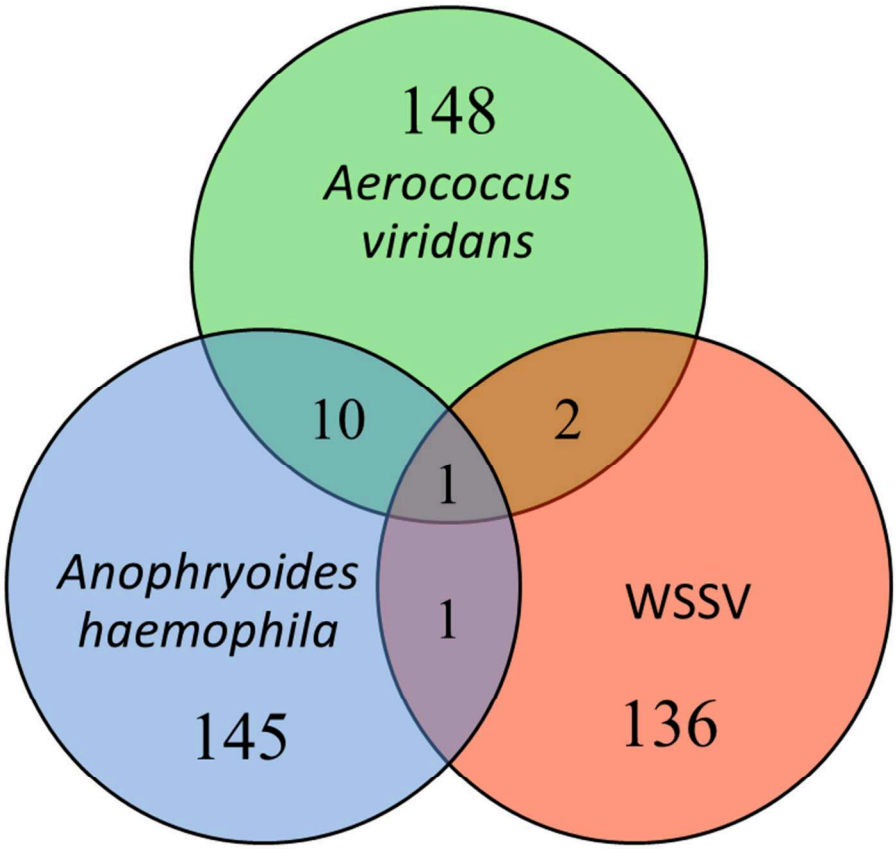

Figure 5.1 Venn diagram of the number of differentially expressed *Homarus americanus* genes in common between two or more listed pathogens as determined by microarray and RT-qPCR analysis.

The unique increase in trypsin 1b expression during all of the immune challenges suggests that it could be very useful as a broadly applicable biomarker of immune activation in response to pathogen infection, and perhaps to lobster health in general. Additional bioinformatic and functional investigation into the functional role of trypsin 1b is warranted.

The expression of ALFHa isoforms is important during bacterial and parasitic infections, however the magnitude and specificity of the individual isoform expression is dependent on the particular pathogen. Moribund *A. viridans* infected lobsters had ALFHa-4 ALFHa-2 and ALFHa-1 expression that was 26.7-fold, 13.4-fold and 12.2-fold higher respectively than its time point control. Moribund *A. haemophila* infected lobsters had ALFHa-4 ALFHa-2 and ALFHa-1 expression that was 8.4-fold, 35.6-fold and 6.73-fold higher respectively than its time point control. This highlights the importance of ALFHa-4 expression during bacterial infection, and ALFHa-2 expression during parasitic infection. Another interesting aspect of ALFHa isoform expression is that in both bacterial and parasitic infections the expression of ALFHa-1 peaked before the lobster became moribund. This isoform specific expression preference within an important immune factor family indicates the interesting, and important, specificity of the lobster immune system.

Although the primary focus of this project was to determine humoral immune molecules, all three challenge trials involved clinical examination of circulating haemocytes to verify *H. americanus* response to the pathogen. In the *A. haemophila* challenge trial there is a large increase in circulating haemocyte concentration in both the control and infection

groups at 24 h and 48 h after injection with either *A. haemophila* in sterile 3% NaCl, or with sterile 3% NaCl alone (Figure 3.1). This increase in circulating haemocytes returns to normal by 1 week and is steady in the control group for the remainder of the trial. The fact that both *A. haemophila* infected, and control animals are affected suggests that the mobilization of haemocytes into the circulation is a response to either the physical insult caused by the needle perforating the membranous exoskeleton, or the injection of 200 μL of 3% NaCl. The fact that this didn't occur during the bacterial or viral challenge experiments could be related to temperature as the *A. haemophila* challenge occurred at 2 ^{o}C while the bacterial and viral challenges occurred at 15 ^{o}C and 20 ^{o}C respectively.

5.6 Future Work

Additional research into the lobster immune response to multiple pathogens from a similar pathogen class will expand our understanding of the specificity of immune response. An excellent experiment would be to use a Gram-negative bacterium such as *Photobacterium indicum* and an avirulent Gram-positive *A. viridans* strain and monitor the immunological response to these microorganisms.

It would also be very interesting to investigate the effect of temperature on lobster immunity. The *A. haemophila* and *A. viridans* experiments took place at 2 ^{o}C and 15 ^{o}C respectively but despite a difference of 13 ^{o}C, the response of several key immune genes such as SAA and the ALFHa family of ALFs was more similar than in response to WSSV at 20 ^{o}C. Perhaps 20 ^{o}C represents an upper limit of lobster immunity where temperature

stress has a significant impact on the ability of a lobster to respond to opportunistic pathogen challenges.

My final avenue of suggested research would be to explore the similarities and differences between male and female lobster immune response. Female lobsters have more physiologically demanding reproductive roles than male lobsters and this would undoubtedly complicate the metabolic needs for immune response. A study of the female lobster immune response will be required before any biomarker for lobster health or disease could be broadly implemented.

5.7 Conclusion

The *H. americanus* bacterial, parasitic and viral immune response studies have clearly demonstrated that lobster research is on the cusp of major advancements in our understanding of lobster immunity. Genomic advancements like microarrays and rapid high-throughput sequencing have changed the way one can investigate and explore the biological sciences. The microarray approach significantly advanced to the understanding of lobster immunity. The finding of differential expression within families of immune factors to different pathogens is especially important. Another important finding was the similarity in innate immune activation between humans and lobsters in the form of SAA expression, which suggests an evolutionary important functional conservation. It will be important to continue the investigation into lobster immunity by looking at a larger number of pathogens from similar pathogen classes, varying temperatures and comparison of male and female lobsters to truly comprehend the breadth and specificity

of the lobster immune response. The discoveries that have been made by these studies provide a strong framework to continue exploring and discovering the subtle nuances that lobster immunology has to offer.

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Appendix A

Table A.1 *H. americanus* genes differentially expressed during *Aerococcus viridans* infection as measured with a microarray at a significance level of $\alpha = 0.005$.

Cluster 1 (16 genes)

Gene Name	Accession #	6h Control	6h Infected	12h Infected	24h Infected	48h Infected	76h Infected	76h Control	p-value
NA	DV771836.1	-5.54	-6.30	-6.59	-6.33	-6.36	-5.44	-5.53	0
NA	EW702608.1	-4.19	-4.55	-4.35	-4.61	-4.97	-3.82	-3.81	0
NA	CN852893.1	-2.05	-2.33	-2.38	-2.64	-3.06	-2.72	-1.96	0.001
NA	DV771094.1	0.89	0.10	0.25	-0.18	-1.41	-1.38	0.34	0.001
thermosome subunit alpha [Culex quinquefasciatus]	EG948653.1	-5.95	-6.31	-6.60	-6.14	-6.39	-5.68	-5.53	0.001
NA	EH116499.1	2.90	2.48	2.51	1.93	0.88	1.46	2.77	0.001
NA	EW703026.1	-3.14	-3.72	-4.22	-4.13	-4.91	-4.77	-3.58	0.001
NA	EX471295.1	-1.08	-0.92	-1.50	-0.68	-1.46	-0.56	-0.05	0.001
NA	EX487861.1	-2.56	-2.54	-2.52	-2.87	-4.12	-4.14	-3.50	0.001
DEAD (Asp-Glu-Ala-Asp) box polypeptide 10 [Mus musculus]	FC555983.1	0.50	-0.61	-0.67	-0.48	-0.88	-0.22	0.85	0.001
NA	DV771834.1	2.28	1.49	2.49	1.56	1.05	1.25	2.84	0.002
NA	FC071822.1	-2.62	-3.11	-3.41	-2.80	-3.35	-2.88	-2.06	0.002
AGAP010729-PA [Tribolium castaneum]	FF277743.1	-1.50	-1.78	-1.76	-1.49	-2.02	-1.19	-1.13	0.002
NA	CN852893.1	-2.33	-3.13	-3.17	-3.24	-3.11	-1.91	-2.56	0.003
transposase [Strongylocentrotus purpuratus]	FD699682.1	-1.02	-1.94	-1.28	-2.23	-2.31	-1.13	-1.16	0.005
NA	FE841399.1	-2.32	-2.89	-3.25	-3.48	-4.20	-2.63	-1.70	0.005

Cluster 2 (53 genes)

Gene Name	Accession #	6h Control	6h Infected	12h Infected	24h Infected	48h Infected	76h Infected	76h Control	p-value
anti-lipopolysaccharide factor 2 [Homarus americanus]	EX568422.1	-2.39	-2.54	-1.93	-1.32	-0.45	0.45	-2.93	0
anti-lipopolysaccharide factor like protein [Marsupenaeus japonicus]	DV774220.1	-1.60	-1.51	-1.30	-0.54	0.30	0.78	-2.06	0
NA	CN852914.1	-0.04	0.18	0.25	0.47	1.12	1.58	0.34	0
NA	FD584806.1	-0.79	-1.03	-1.36	-0.95	-0.28	0.35	-0.51	0
reticulocalbin [Tribolium castaneum]	EY290811.1	-1.19	-1.04	-0.76	0.02	0.81	0.75	-1.26	0
chromatin assembly factor i P60 subunit [Aedes aegypti]	EX487868.1	1.04	1.55	1.51	2.08	1.99	2.47	1.62	0.001
D polymerase epsilon subunit B [Xenopus laevis]	CN951983.1	0.96	1.31	1.34	2.26	3.59	4.14	1.21	0.001
NA	EH116331.1	0.15	0.22	-0.42	0.38	1.15	1.50	-0.14	0.001
NA	EH035492.1	0.78	1.39	1.42	2.42	3.52	4.30	1.86	0.001
NA	EW998161.1	-1.14	0.19	0.71	0.99	0.78	2.39	-0.73	0.001
NA	FE659960.1	1.04	1.00	1.13	1.60	1.89	2.56	1.22	0.001
NA	EY117097.1	0.33	0.92	0.76	1.55	2.67	3.61	1.03	0.001
NA	FD585073.1	-0.30	0.04	0.82	1.05	1.78	2.58	0.01	0.001
Solute carrier family 43 member 3 (Protein FOAP-13) [Equus caballus]	EX471748.1	-0.82	-0.74	-0.88	-0.41	0.26	0.73	-0.54	0.001
acute phase serum amyloid A (SAA) [Oncorhynchus mykiss]	CN949986.1	-2.12	-1.71	-0.51	-0.67	-0.16	0.33	-2.38	0.002
disrupted in renal carcinoma 2 [Xenopus tropicalis]	FD584652.1	-0.45	-0.51	-0.64	-0.43	0.34	0.96	-0.58	0.002
NA	DV774124.1	-1.05	-1.22	-1.27	-0.49	0.35	1.30	-0.75	0.002
NA	EW702788.1	0.08	-0.45	-0.02	0.94	1.19	1.59	0.12	0.002
NA	DV773899.1	-2.85	-2.68	-2.80	-2.59	-2.47	-1.27	-2.28	0.002
NA	DV774565.1	1.80	2.59	2.52	3.12	4.19	5.10	2.41	0.002
NA	CN852986.1	-1.91	-1.15	-1.05	-0.79	0.86	1.18	-1.71	0.002
NA	EY291253.1	-0.52	-0.06	-0.38	0.46	0.57	0.78	-1.98	0.002
NA	EV781710.1	0.93	0.92	1.05	0.95	1.48	1.71	0.62	0.002
thioesterase superfamily member 2 [Taeniopygia guttata]	EH401500.1	1.79	2.41	2.94	3.41	4.21	4.73	2.55	0.002
thioredoxin 1 [Litopenaeus vannamei]	FD699182.1	-3.66	-3.81	-3.53	-3.10	-2.17	-1.99	-3.98	0.002
trypsin -1b [Homarus americanus]	EF095144	-4.30	-4.01	-4.18	-3.55	-1.32	-1.04	-3.83	0.002
"C2H2 finger domain protein, [Aspergillus clavatus NRRL 1]"	CN951780.1	0.23	0.53	0.96	1.32	2.56	3.01	0.25	0.003
modifier of mdg4 [Culex quinquefasciatus]	FE043886.1	0.29	0.41	0.60	1.48	2.76	3.47	0.44	0.003
NA	CN950336.1	-2.90	-2.40	-2.25	-1.80	-1.75	-0.97	-2.33	0.003
NA	FF277966.1	1.81	2.41	2.25	2.76	4.00	4.80	1.90	0.003
NA	DV774795.1	-1.15	-0.82	-0.28	0.10	1.82	2.97	-1.15	0.003
NA	EY116867.1	1.96	2.51	2.23	2.91	3.65	4.24	2.20	0.003
NA	FE535841.1	1.35	1.21	1.38	1.50	2.11	2.72	1.22	0.003
NA	EX568247.1	-3.16	-3.19	-2.45	-1.85	-1.20	-0.35	-2.97	0.003
NA	CN852704.1	2.65	3.06	3.12	3.60	4.84	5.17	3.07	0.003
NA	FD699637.1	1.47	1.49	1.94	2.22	4.23	4.68	0.99	0.003
NA	EH401872.1	-4.36	-4.72	-4.46	-4.22	-3.46	-2.94	-4.14	0.003
ribulose-5-phosphate 4-epimerase-like protein [Branchiostoma belcheri tsingtaunese]	EX471450.1	-5.75	-5.88	-5.39	-5.48	-4.92	-4.32	-5.56	0.003
SCP-related protein [Bombyx mori]	CN854395.1	-1.42	-0.85	-0.50	-0.11	0.68	1.17	-1.03	0.003
"zinc metallopeptidase, STE24 homolog [Danio rerio]"	FC556423.1	0.32	0.47	0.63	0.79	2.22	2.43	0.32	0.004
anti-lipopolysaccharide factor 1 [Homarus americanus]	FF277612.1	-3.56	-3.36	-2.88	-1.55	-1.29	-1.42	-4.34	0.004
arginine\serine-rich splicing factor [Nasonia vitripennis]	CN950265.1	1.98	2.30	2.47	3.28	4.21	4.69	1.85	0.004
LOC540367 protein [Equus caballus]	EG949422.1	1.12	1.86	1.94	2.27	3.41	4.06	1.41	0.004
NA	FD467941.1	-1.53	-1.36	-1.15	-0.76	0.02	0.99	-1.63	0.004
NA	FD468205.1	1.96	2.66	1.96	2.76	4.16	4.66	2.09	0.004
conserved hypothetical protein [Culex quinquefasciatus]	FD425527.1	-0.09	-0.69	0.29	0.79	1.37	1.75	0.33	0.005
glucosyl\glucuronosyl transferases [Tribolium	EG949312.1	1.14	1.36	1.26	1.73	3.39	3.87	0.93	0.005

castaneum]									
NA	EX486482.1	1.77	2.38	2.16	2.66	3.82	4.32	2.31	0.005
NA	EW702937.1	2.80	3.09	3.54	3.98	5.30	5.92	2.75	0.005
NA	CN951986.1	0.87	0.92	0.13	0.75	1.90	1.67	0.66	0.005
NA	FE535686.1	-3.87	-3.41	-3.14	-3.02	-2.05	-1.81	-3.67	0.005
NA	EH401488.1	-4.44	-4.90	-4.52	-4.55	-3.59	-2.96	-4.63	0.005
predicted protein [Monosiga brevicollis MX1]	FE535585.1	0.77	1.05	1.32	1.26	1.78	1.82	0.78	0.005

Cluster 3 (31 genes)

Gene Name	Accession #	6h Control	6h Infected	12h Infected	24h Infected	48h Infected	76h Infected	76h Control	p-value
GF23818 [Drosophila ananassae]	FE535102.1	0.74	0.97	0.09	1.42	1.58	1.80	1.19	0
Hypothetical protein CBG12045 [Caenorhabditis briggsae]	FD699211.1	0.69	1.26	0.44	1.43	1.48	2.03	1.28	0
NA	CN951234.1	1.32	1.80	1.46	2.47	2.37	2.87	1.66	0
NA	FF277751.1	1.42	1.60	1.09	1.92	1.98	2.41	1.72	0
NA	EG948945.1	1.46	1.31	1.27	2.17	2.20	2.21	1.67	0
NA	FE044260.1	-0.07	0.42	-0.35	0.88	0.94	1.57	0.55	0
NA	CN853978.1	-0.54	-0.03	-0.97	0.18	0.50	0.63	-0.22	0
s-adenosyl-methyl transferase mraw [Tribolium castaneum]	EX487535.1	1.35	1.38	0.77	2.13	2.33	2.01	1.49	0
copper\zinc superoxide dismutase [Macrobrachium rosenbergii]	FE044177.1	-2.03	-1.18	-1.74	-0.47	-0.79	0.35	-1.29	0.001
hexokinase [Culex quinquefasciatus]	EH401720.1	0.56	1.53	0.57	1.52	1.96	1.93	0.81	0.001
MRP-like ABC transporter [Arabidopsis thaliana]	EY116994.1	2.51	3.21	2.76	3.71	3.72	3.15	3.27	0.001
NA	EX486823.1	0.42	1.40	0.57	1.60	2.23	1.34	0.69	0.001
NA	DV772830.1	1.74	2.38	1.69	2.76	2.77	2.42	2.03	0.001
Nuclear transcription factor Y subunit beta (CAAT-box D-binding protein subunit B) [Apis mellifera]	FD699580.1	0.11	0.97	0.76	1.78	2.03	2.11	1.00	0.001
NA	FD467939.1	2.61	2.91	2.61	3.76	3.52	3.95	3.21	0.002
NA	FD482987.1	1.42	1.88	1.59	2.55	2.45	2.48	2.01	0.002
NA	EH117053.1	1.34	2.06	1.67	2.30	2.74	2.28	1.85	0.002
NA	FC556036.1	1.23	1.84	1.33	2.15	2.32	2.32	2.00	0.002
NA	DV774489.1	1.73	2.77	1.39	2.94	2.78	3.04	2.26	0.003
NA	FF278058.1	1.77	1.81	1.65	2.63	2.82	2.30	2.35	0.003
ankyrin-repeat protein Nrarp [Gallus gallus]	FE044078.1	0.02	1.01	0.23	1.47	1.49	1.24	0.62	0.004
CG9448 CG9448-PA [Tribolium castaneum]	EX486524.1	0.34	-0.28	-0.51	0.79	0.37	0.82	0.07	0.004
NA	DV771264.1	0.87	1.04	0.68	1.81	1.52	1.86	1.33	0.004
NA	FD483459.1	2.28	3.27	2.57	3.54	3.30	3.38	2.95	0.004
spermine synthase [Tribolium castaneum]	EG948805.1	1.53	1.14	-0.65	3.03	2.14	2.49	2.09	0.004
"SYF2 homolog, R splicing factor (S. cerevisiae) [Strongylocentrotus purpuratus]"	FD483713.1	-0.69	-0.23	-1.34	0.58	1.26	0.35	0.42	0.005
NA	FD585383.1	1.90	2.65	2.14	2.72	2.99	2.83	2.20	0.005
NA	FD467517.1	1.39	1.94	1.33	2.40	2.12	2.40	2.30	0.005
NA	FF277987.1	0.34	0.57	-0.18	1.09	1.23	1.13	0.61	0.005
NA	FD585399.1	1.64	1.93	1.26	1.87	2.19	1.86	1.47	0.005
NA	CN951939.1	0.66	2.15	-0.04	2.69	2.35	2.00	2.08	0.005

Cluster 4 (48 genes)

Gene Name	Accession #	6h Control	6h Infected	12h Infected	24h Infected	48h Infected	76h Infected	76h Control	p value
anti-lipopolysaccharide factor 2 [Homarus americanus]	FD483938.1	-4.32	-3.40	-2.91	-1.64	-1.35	-1.74	-4.96	0
chordin [Lytechinus variegatus]	EX487273.1	1.64	2.35	2.36	2.60	2.92	2.67	1.98	0
Exosome complex exonuclease RRP40 (Ribosomal R processing protein 40) [Canis familiaris]	EG949026.1	0.70	1.54	1.66	2.12	2.44	2.07	1.37	0
hypothetical protein NEMVEDRAFT_v1g219856 [Nematostella vectensis]	FD584946.1	2.64	3.50	3.29	4.19	4.04	3.83	3.39	0
mannose-binding protein [Pacifastacus leniusculus]	CV222270.1	2.38	2.66	2.65	3.25	3.49	3.12	2.48	0
NA	FC555919.1	0.19	1.41	1.34	2.10	1.43	1.85	1.42	0
NA	DV774700.1	2.05	2.59	2.93	3.36	3.22	3.05	2.56	0
NA	FD699834.1	0.17	1.70	1.28	2.32	1.50	1.84	1.62	0
NA	FE535547.1	2.11	3.26	2.96	3.23	3.42	2.50	2.04	0
NA	EW702735.1	1.27	2.14	1.73	2.28	2.45	2.17	1.74	0
ring finger protein 7 [Gallus gallus]	EH116259.1	1.48	2.39	2.36	2.71	3.01	2.70	2.12	0
solute carrier family 35 member B1 [Acyrthosiphon pisum]	FE535241.1	-0.75	1.49	1.07	1.31	1.28	1.35	0.81	0
"supercoiling factor CG9148-PA, isoform A [Drosophila melanogaster]"	CN853531.1	-0.44	1.05	1.03	1.49	1.91	1.94	0.97	0.001
"triacylglycerol lipase, pancreatic [Aedes aegypti]"	FE043708.1	-0.74	-0.17	0.14	0.32	-0.11	-0.94	-0.92	0.001
Armadillo [Achaearanea tepidariorum]	FE535061.1	1.89	2.66	2.60	2.87	2.77	3.22	2.34	0.001
Fumarylacetoacetase (FAA) (Fumarylacetoacetate hydrolase) (Beta-diketonase)	CN853326.1	0.94	1.55	1.29	1.74	1.82	1.75	1.31	0.001
NA	FF277973.1	2.16	3.44	3.16	3.86	4.04	3.55	2.90	0.001
NA	FF277360.1	2.25	2.94	2.89	3.33	3.30	3.16	2.46	0.001
NA	FF277497.1	-1.98	1.30	0.91	1.86	1.33	1.22	1.44	0.001
NA	FD483203.1	1.35	1.86	1.98	2.44	2.65	2.27	1.66	0.001
NA	CN950045.1	1.78	2.25	2.06	2.55	2.70	2.17	1.99	0.001
unnamed protein product [Tetraodon nigroviridis]	CN853102.1	1.59	2.16	2.45	2.64	2.66	2.50	2.18	0.001
"CG15111 CG15111-PB, isoform B [Drosophila melanogaster]"	CN854069.1	2.63	3.07	3.18	3.74	3.33	3.33	3.13	0.002
Chromatin assembly factor 1 subunit CG4236-PA [Apis mellifera]	FD585467.1	0.20	1.52	1.01	2.12	1.13	1.88	0.66	0.002
microtubule-associated protein [Nasonia vitripennis]	DV773068.1	-0.28	1.16	0.72	1.52	1.59	1.44	1.09	0.002
NA	CN852661.1	2.16	3.03	2.53	3.17	3.40	3.27	2.68	0.002
NA	FD699206.1	1.78	2.82	2.39	2.79	3.09	3.18	2.37	0.002

NA	CN950902.1	-2.31	-0.95	-0.88	-1.06	-1.06	-1.50	-2.29	0.002
coiled-coil protein [Geobacillus kaustophilus HTA426]	FF277563.1	1.09	2.08	1.42	2.64	2.03	2.19	1.73	0.003
conserved hypothetical protein [Tribolium castaneum]	FD584626.1	0.37	1.28	1.23	1.79	1.91	1.55	1.02	0.003
hypothetical protein LOC549079 [Xenopus tropicalis]	EX487220.1	-0.38	0.47	0.32	0.62	0.97	0.42	0.23	0.003
nucleotide binding protein 1-like protein [Strongylocentrotus purpuratus]	EX471729.1	0.45	1.91	1.79	2.10	2.15	1.78	1.47	0.003
segmentation protein cap'n'collar [Aedes aegypti]	EH116633.1	-0.81	0.34	0.36	0.20	0.16	0.86	0.37	0.003
"beta-1,4-mannanase precursor [Cryptopygus antarcticus]"	FE535695.1	0.65	1.42	1.81	2.53	2.18	2.02	1.57	0.004
"Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit, delta isoform (PI3-kinase p110 subunit delta) (PtdIns-3-kinase"	FF278128.1	1.39	2.90	2.31	3.13	3.05	2.72	2.78	0.004
alkylglycerone phosphate synthase [Xenopus laevis]	CN853043.1	1.71	2.37	2.09	2.90	2.89	2.42	2.22	0.004
hemocyanin subunit 3 [Cancer magister]	CN950724.1	-2.72	-0.98	-0.90	-1.82	-4.54	-4.43	-4.85	0.004
NA	FE659560.1	-0.51	0.74	0.48	0.97	1.03	1.38	0.95	0.004
thrombospondin [Acyrthosiphon pisum]	CN854443.1	0.64	1.28	0.90	1.39	1.97	1.40	0.82	0.004
U3 snoRNP protein Utp14 (predicted) [Schizosaccharomyces pombe 972h-]	FE043944.1	0.01	1.03	0.52	1.60	1.22	1.42	0.86	0.004
"arginyl-tR synthetase 2, mitochondrial [Rattus norvegicus]"	FF277899.1	2.26	2.67	2.37	3.01	2.75	2.30	1.92	0.005
"WD repeat domain 43, like [Apis mellifera]"	EY117136.1	0.58	1.83	2.19	2.96	2.30	2.75	1.76	0.005
cyclophilin [Nasonia vitripennis]	EY291251.1	1.73	2.10	2.02	2.44	2.71	2.16	1.80	0.005
msta [Culex quinquefasciatus]	CN854016.1	1.41	2.05	2.23	2.43	2.76	2.23	1.57	0.005
receptor tyrosine phosphatase type r2a [Culex quinquefasciatus]	DV773403.1	1.81	2.44	2.01	2.67	2.89	2.45	1.81	0.005
Rho guanine nucleotide exchange factor 4 [Gallus gallus]	FE659412.1	0.50	1.84	1.52	2.64	2.06	2.17	1.83	0.005
serine proteinase inhibitor [Procambarus clarkii]	FD585298.1	-0.11	1.78	1.12	2.29	1.77	1.18	1.13	0.005
transportin [Tribolium castaneum]	FD483411.1	0.94	1.59	1.51	1.81	2.24	2.09	1.21	0.005

Appendix B

Table B.1. Forward and reverse primers used in RT-qPCR experiments during the *Anophryoides haemophila* infection trial.

Gene	Accession #	Forward	Reverse	Annealing Temperature (°C)	Reaction Efficiency (%)	# Step Reaction
ALFHa-1	EU625516	CAGTCGTTCTGGTGTTGTTGGGAA	TTGTTGGGCATCCCTCTCGGTTAT	65	99	3
ALFHa-2	FC556430	AGACTACCACTGACTTCGTGAGGA	TCTCGGGATGATCCGTTAACACCT	65	95	3
ALFHa-4	DV772634	ACAAGACAAGAGAATGCGTCCCTC	TGATAGCTTGTCCACGAAGGCTG	65	93	3
SAA	EH116055	TACCACTACCAGCACTCATCACCT	TCAAACACAGAGAATAGGCACGGG	59	99	3
C-type lectin receptor	CN852574	AGGCGTTCTCCTTTACCTGGTTGA	ACACAGGCTTCCCACCTAACATCT	67	93	3
Pseudohaemocyanin-2 precursor	CN952339	GCCAAACTGAAGGCCATGGAAGAT	ATGTTGGGTCTTGACCTCCTGCAT	67	89	3
Phosphoenolpyruvate carboxykinase 1	DV773064	TTGGTTCCAACATTGCCAGACGTG	AAGCGATATCATCACCCACGCACT	64	99	3
Trypsin 1a	EV781656	CGTCCAATGTTAAGCGTCATGCCA	TTCCAAGTCTTGCCCGTAGACACA	65	93	3
Trypsin 1b	EF095144	AGCTACCGCAACATCGGCTATACT	ATGAAGTAGCGGTTGTCAGCTCCA	67	92	3
Thioredoxin	FD699182	TTTGACAAGCAGTTGGCTGATGCC	TCCACGTCCACCTTCAAGAACACT	65	93	3
Coagulation factor VIII-associated protein	FD699377	TTAGGGCGGCCATCTGAAGCATTA	TCCAGTGAGTGGTTGTTCAGCAGT	67	93	3
Lava lamp protein	CN853553	ATTGTTATTCTCCTGCCCA	TGGACCAAAAGTTAGCTGAA	57	108	3

Table B.2 *H. americanus* genes differentially expressed during *Anophryoides haemophila* infection as measured with a microarray at a significance level of $\alpha = 0.005$.

Cluster 1 (8 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value
condensin II non-SMC subunit [Xenopus laevis]	FF277113	0.000	12.184	12.396	7.666	4.175	8.380	8.600	11.791	8.264	31.853	0.001
serine proteinase inhibitor [Pacifastacus leniusculus]	CN951034	0.000	0.326	0.206	-0.112	0.195	0.237	0.181	0.128	0.064	0.329	0.005
delta-aminolevulinate dehydratase [Nasonia vitripennis]	FD584953	0.000	-0.066	0.124	-0.186	0.024	-0.192	0.018	0.069	-0.016	0.072	0.005
NA	CN951763	0.000	0.294	0.384	0.057	0.562	0.576	0.088	0.713	0.269	0.442	0.002
NA	CN950679	0.000	-0.952	0.177	-0.933	-0.590	-0.120	-0.569	0.107	-0.441	-0.117	0.002
NA	FE535737	0.000	-0.104	0.311	0.086	0.227	0.221	-0.248	0.288	-0.133	0.733	0.005
NA	EW703070	0.000	-0.349	-0.147	-0.007	-0.122	0.096	-0.131	0.430	-0.086	0.494	0.001
NA	CN950093	0.000	-0.159	0.045	0.214	0.257	0.117	0.271	0.198	0.055	0.504	0.001

Cluster 2 (20 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value
supervillin isoform 1 [Apis mellifera]	EY290972	0.000	-0.339	0.865	0.238	0.176	-0.249	0.879	0.174	1.105	0.029	0.005
NA	FD467894	0.000	-0.717	-0.474	-0.116	-0.285	-0.390	-0.028	-0.341	0.141	-0.689	0.003
zinc finger protein 544 [Danio rerio]	FD585320	0.000	-0.280	0.390	0.027	-0.054	-0.221	0.304	-0.019	0.066	-0.081	0.003
histone aminotransferase 1 [Apis mellifera]	EX471046	0.000	-0.847	-0.124	-0.481	-0.500	-0.350	0.228	-0.182	0.357	-0.054	0.001
GH14865 [Drosophila grimshawi]	EG948858	0.000	-0.652	-0.223	-0.269	-0.221	-0.387	0.295	-0.371	0.190	-0.294	0.002
CGI-41 protein [Gallus gallus]	FD467684	0.000	-0.527	-0.086	-0.017	-0.593	-0.170	0.521	-0.058	0.459	-0.131	0.002
NA	FC556512	0.000	-0.460	0.168	-0.001	-0.186	0.043	0.146	0.144	0.931	-0.396	0.002
NA	EW997965	0.000	-0.451	0.322	-0.025	0.039	-0.159	0.744	-0.439	0.305	-0.376	0.001
NA	EW703041	0.000	-0.223	-0.047	-0.180	-0.248	-0.186	0.125	-0.285	-0.180	-0.004	0.005
NA	EX487198	0.000	-0.963	-0.259	-0.208	-0.643	-0.367	0.211	-0.408	0.905	-0.608	0.000
NA	EY116677	0.000	-0.280	0.186	0.459	0.065	0.287	0.409	0.264	0.947	-0.048	0.000
NA	EH401779	0.000	-0.281	0.285	0.060	-0.097	-0.190	1.380	0.235	0.716	-0.425	0.005
NA	FE659782	0.000	-0.324	0.058	-0.073	-0.299	-0.548	-0.134	-0.251	-0.177	-0.170	0.003
elongation factor Tu [Bombyx mori]	CN950782	0.000	-0.309	-0.121	-0.306	-0.309	-0.160	-0.108	-0.107	0.366	-0.718	0.004
acylglycerol kinase [Mus musculus]	CN853540	0.000	-0.402	-0.422	-0.805	-0.436	-0.722	-0.528	-0.673	-0.257	-1.065	0.004
Phagocyte signaling-impaired protein [Tribolium castaneum]	EH401578	0.000	-0.996	-0.386	-0.230	-0.587	-0.752	-0.226	-0.493	0.764	-0.516	0.004
Chaperone protein dnaJ	EX471373	0.000	-1.039	-0.134	0.187	-0.585	-0.360	-0.046	-0.061	1.300	-0.912	0.000
CG12012 CG12012-PA [Drosophila melanogaster]	FE841154	0.000	0.005	-0.165	0.231	-0.164	0.200	0.412	0.327	0.405	-0.079	0.004
NA	EX471533	0.000	-0.720	-0.084	-0.583	-0.148	0.138	0.115	0.120	-0.112	-0.670	0.005
NA	FD467623	0.000	-1.097	-0.648	0.079	-0.224	-0.311	-0.063	-0.204	0.497	-0.528	0.005

Cluster 3 (20 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value
hemocyanin subunit 3 [Cancer magister]	CN950724	0.000	0.736	0.778	0.477	0.624	-1.417	-0.839	-2.650	-1.001	-1.228	0.000
hemocyanin alpha subunit [Homarus americanus]	FD699910	0.000	0.816	2.431	0.999	1.005	-0.843	-0.258	-1.895	-0.681	-1.140	0.000
fatty Acid CoA Synthetase family member (acs-2) [Caenorhabditis elegans]	CN950542	0.000	-0.716	-0.737	-0.884	-0.867	-0.686	-0.654	-1.127	-1.032	-0.954	0.003
ENSANGP00000020847 [Nasonia vitripennis]	CN950729	0.000	0.611	0.817	0.541	0.546	-0.071	-0.116	-0.502	-0.037	-1.003	0.001
BTB-protein-VII CG11494-PA, isoform A [Apis mellifera]	FF277123	0.000	0.232	0.378	0.412	0.062	-0.849	-1.117	-1.249	-1.695	-1.300	0.000
NA	FC071443	0.000	-0.266	-0.089	-0.255	-0.202	-0.333	-0.508	-0.700	-0.172	-0.764	0.004
NA	EW702841	0.000	0.119	0.324	-0.293	0.334	-0.876	-0.652	-1.184	-0.223	-0.771	0.004
vitelline membrane outer layer protein I-like protein [Pacifastacus leniusculus]	EG949216	0.000	0.913	0.643	0.511	0.210	-0.082	-0.048	-0.311	-1.526	-0.552	0.000
nucleotide pyrophosphatase-like protein [Spinacia oleracea]	CN951088	0.000	0.422	0.683	0.304	0.307	-0.499	-0.460	-0.583	-1.188	-0.913	0.000
hemocyanin alpha-subunit [Homarus americanus]	CN854285	0.000	0.704	0.933	0.774	0.511	-0.691	-0.252	-1.182	-0.686	-0.551	0.002
Pseudohemocyanin-2 precursor	EX487794	0.000	0.036	1.115	0.717	-0.341	-1.414	-1.414	-2.575	-3.022	-2.473	0.000
Pseudohemocyanin-2 precursor	CN952339	0.000	0.438	0.853	0.755	0.036	-1.294	-1.605	-2.103	-2.871	-2.040	0.001
GI21440 [Drosophila mojavensis]	CN853239	0.000	0.204	0.238	0.410	0.230	0.298	-0.648	0.254	-0.002	-0.016	0.002
C-type lectin receptor [Portunus pelagicus]	CN852574	0.000	1.769	1.813	1.430	0.688	0.233	-0.012	0.096	0.162	-0.296	0.000
Cap1 CAP, adenylate cyclase-associated protein 1 [Danio rerio]	CN951092	0.000	-0.321	0.232	0.272	-0.358	-1.055	-2.472	-1.398	-1.316	-2.100	0.004
NA	CN854017	0.000	0.320	0.271	-0.115	-0.021	-0.928	-0.849	-1.236	-0.393	-0.871	0.003
NA	CN950902	0.000	0.193	0.213	0.183	0.258	-0.352	-0.451	-0.730	-1.030	-0.447	0.002
NA	FC556653	0.000	0.007	0.703	-0.316	0.119	-0.922	-0.722	-0.943	-0.949	-0.927	0.000
heat shock protein 70 [Homarus americanus]	DV774474	0.000	1.134	0.664	0.461	0.041	0.200	0.569	0.389	0.513	0.357	0.003
Choline transporter-like protein 1 (Solute carrier family 44 member 1) (CD92 antigen)	CN852546	0.000	-0.265	-1.109	-0.816	-0.702	-0.478	-1.111	-1.107	-0.965	-0.972	0.001

Cluster 4 (18 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value

thioredoxin 2 [Bos taurus]	FC556011	0.000	-0.263	-0.329	-0.312	0.295	-0.060	-0.441	-0.148	-0.160	-0.043	0.004
splicing factor pTSR1, [Tribolium castaneum]	CN950611	0.000	-0.487	-0.625	-0.448	-0.295	-0.590	-0.638	-0.099	0.560	-0.549	0.000
beta1,4 mannosyltransferase [Nasonia vitripennis]	CN854471	0.000	-0.310	-0.426	-0.154	-0.169	-0.195	-0.585	-0.332	-0.041	-0.221	0.001
NA	DV774685	0.000	-0.487	-0.377	-0.122	-0.164	-0.116	-0.374	0.127	0.161	-0.060	0.000
hypothetical protein TRIADDRAFT_60866 [Trichoplax adhaerens]	EY117263	0.000	0.030	-0.270	0.386	0.097	0.049	-0.166	0.097	0.347	-0.166	0.002
beta-tubulin cofactor D [Tribolium castaneum]	CN951947	0.000	-0.553	-0.304	-0.389	-0.557	-0.358	-0.571	-0.154	-0.030	-0.233	0.000
Transcription initiation protein SPT3 homolog (SPT3-like protein)	FE043748	0.000	-0.313	-1.078	-0.407	-0.962	-0.230	-0.243	-0.289	-0.043	-0.606	0.001
NA	EX568435	0.000	-0.795	-0.444	-0.770	-1.011	-0.600	-1.422	-0.680	0.067	-1.020	0.000
NA	CN853427	0.000	-0.028	-0.508	-0.029	0.047	-0.050	0.111	0.115	0.407	0.040	0.003
NA	FD699722	0.000	0.210	-0.745	-0.660	-0.808	-0.521	-1.000	-0.871	-0.085	-0.939	0.001
NA	DV772826	0.000	0.164	-1.558	0.145	0.702	0.103	-0.335	0.032	0.623	1.083	0.000
NA	CN853981	0.000	-0.086	-0.766	0.136	0.160	0.256	0.403	0.411	0.738	0.490	0.001
NA	EW702776	0.000	-0.426	-0.491	-0.170	-0.045	-0.160	0.186	0.056	0.263	0.429	0.005
NA	CN949956	0.000	-1.623	-1.167	-0.994	-2.432	-1.091	-1.237	-1.949	-0.401	-1.034	0.005
NA	EY116792	0.000	-1.418	-1.069	-0.716	0.271	-0.604	-0.769	-0.737	-0.354	-0.375	0.000
kaptin (actin binding protein) [Homo sapiens]	CN952074	0.000	0.130	-0.609	-0.542	-0.304	-0.178	-0.567	-0.369	0.253	-0.210	0.003
NA	CN853667	0.000	-0.439	-0.268	-0.919	-0.663	-0.294	-0.478	-0.497	-0.105	-0.424	0.002
NA	EX568298	0.000	-0.297	-0.777	-0.332	-0.556	-0.314	0.047	-0.408	-0.116	-0.513	0.005

Cluster 5 (31 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value
segmentation protein cap'n'collar [Aedes aegypti]	EH116633	0.000	0.686	0.207	0.229	-0.212	0.857	-0.626	0.247	0.529	-0.236	0.005
ribosomal protein L30 [Gallus gallus]	EY291074	0.000	-0.043	0.365	0.151	0.162	0.557	-0.009	0.211	6.838	-0.197	0.001
furry homolog-like, partial [Acyrthosiphon pisum]	FF277622	0.000	0.160	-0.363	-0.272	0.074	0.468	0.549	0.333	5.582	0.082	0.004
CG10077-PA, isoform A, partial [Apis mellifera]	DV774405	0.000	0.592	0.079	0.273	0.233	0.311	0.325	0.815	0.808	-0.191	0.004
NA	FD483732	0.000	0.338	-0.143	-0.095	0.234	0.239	0.579	0.171	1.087	-0.127	0.002
NA	FD699443	0.000	-0.152	0.212	0.170	-0.132	0.055	0.313	0.270	0.829	0.083	0.003
hypothetical protein TRIADDRAFT_55664 [Trichoplax adhaerens]	FE535204	0.000	0.323	-0.051	0.160	-0.162	0.322	-0.082	0.597	0.512	-0.441	0.000
D polymerase delta subunit 3 [Oreochromis mossambicus]	CN853982	0.000	0.100	-0.152	-0.158	-0.193	-0.047	-0.044	-0.225	0.168	-0.302	0.005
NA	FD483929	0.000	0.202	-0.119	-0.169	0.144	0.287	-0.085	-0.035	0.649	-0.577	0.005
NA	EX471558	0.000	0.358	0.468	0.666	0.262	0.616	0.876	0.840	2.507	0.281	0.003
NA	EG949353	0.000	0.635	0.119	0.635	-1.540	0.572	0.939	0.906	1.475	0.787	0.003
NA	CN951323	0.000	0.198	0.111	0.356	0.264	0.484	0.669	0.977	1.387	-0.161	0.004
NA	DV771258	0.000	0.074	0.405	0.716	0.716	0.525	0.356	0.570	1.093	0.201	0.001
NA	FD483115	0.000	-0.676	-0.214	-0.610	-0.435	-0.194	-0.464	0.072	1.173	-0.891	0.000
NA	FD467484	0.000	0.407	0.423	0.285	0.000	0.569	0.192	0.257	1.349	0.010	0.002
NA	EX568307	0.000	-0.114	0.013	0.065	0.080	0.128	0.428	-0.662	2.929	-0.281	0.000
NA	CN951732	0.000	0.604	0.615	0.254	0.694	0.512	0.542	0.612	1.287	0.700	0.001
NA	FE043873	0.000	-0.123	0.318	0.193	0.016	0.143	-0.320	0.552	1.080	0.137	0.003
NA	CN854493	0.000	-0.138	0.345	0.061	-0.020	0.059	0.411	0.362	1.960	0.024	0.001
NA	EX471757	0.000	-0.349	0.264	-1.261	0.037	-0.276	-0.131	0.057	17.023	-0.033	0.003
NA	DV772011	0.000	0.386	0.211	0.610	0.139	0.081	0.229	0.230	2.267	0.012	0.000
NA	CN853953	0.000	-0.515	0.129	-0.147	-0.226	-0.127	-0.278	0.133	0.406	0.033	0.000
ras-related protein 2 [Bombyx mori]	EX568086	0.000	0.318	0.683	0.381	0.493	0.363	0.313	0.376	1.034	0.344	0.001
allantoicase [Strongylocentrotus purpuratus]	FD584487	0.000	0.549	0.550	-0.216	0.264	-0.148	-0.026	0.019	0.722	-0.491	0.004
CG5937-PA [Apis mellifera]	EG948584	0.000	0.127	0.574	0.544	0.124	0.378	0.259	0.195	1.270	-0.367	0.003
solute carrier family 7, (cationic amino acid transporter, y+ system) member 11 [Homo sapiens]	FD584763	0.000	-0.226	0.533	0.236	0.036	0.294	0.446	-0.321	1.482	0.323	0.003
mCG9285, partial [Sus scrofa]	CN951377	0.000	-0.033	0.183	0.142	-0.174	-0.220	-0.766	-0.191	0.586	-0.573	0.000
NA	DV772249	0.000	-0.183	0.003	-0.023	-0.350	-0.059	-0.465	-0.758	0.409	-0.576	0.005
NA	EX568659	0.000	0.192	0.432	0.306	0.180	0.422	0.368	0.278	0.678	-0.059	0.003
NA	FE840942	0.000	-0.489	-0.016	0.141	-0.219	0.045	-0.207	0.302	0.776	-0.060	0.000
ankyrin repeat and sterile alpha motif domain containing 3 [Mus musculus]	FD699004	0.000	-0.351	-0.210	0.202	-0.281	0.001	-0.165	-0.344	0.329	-0.221	0.003

Cluster 6 (30 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value
NA	EY291383	0.000	-0.477	-0.756	-0.175	-0.245	-0.056	0.499	0.116	-1.723	0.030	0.000
NA	FE841116	0.000	-0.507	-0.572	-0.526	-0.456	0.107	-0.431	-0.263	-1.368	-0.528	0.005
peptidyl-tR hydrolase 2 [Strongylocentrotus purpuratus]	FE535629	0.000	-0.857	-0.498	-0.168	0.079	-0.356	-0.336	-0.216	-1.155	-0.661	0.005
Ubiquitin-like 1 activating enzyme E1B (SUMO-1 activating enzyme subunit 2) (Anthracycline-associated resistance ARX) isoform 1	DV773484	0.000	-0.487	-0.854	-0.443	-0.366	-0.296	-0.415	-0.760	-1.201	-0.265	0.001
Transmembrane protein 98 (Protein TADA1) [Tribolium castaneum]	FC555932	0.000	0.082	-0.041	0.109	0.426	0.535	0.034	0.410	-0.906	0.513	0.004
N-acetylneuraminic acid phosphate synthase [Homo sapiens]	FC071233	0.000	0.130	0.105	-0.017	0.122	0.065	0.294	0.160	-1.477	0.008	0.003
HEAT repeat containing 6 [Mus musculus]	EX487526	0.000	-0.540	-0.802	-0.533	-0.695	-0.349	-0.294	-0.217	-1.263	-0.722	0.005
CG14972-PA [Apis mellifera]	FD467977	0.000	0.821	0.198	0.151	0.438	0.704	-0.310	0.058	-1.410	-0.229	0.004
ATP-dependent R helicase [Aedes aegypti]	EH401673	0.000	-0.067	-0.473	0.193	0.274	0.203	0.217	0.210	-1.058	0.412	0.005

AGAP002691-PA [Tribolium castaneum]	CN852821	0.000	-0.386	-0.280	-0.757	-0.423	-0.535	-0.897	-0.457	-1.059	-0.507	0.001
sphingomyelin phosphodiesterase 4, neutral membrane (neutral sphingomyelinase-3) [Equus caballus]	CN854459	0.000	-0.270	-0.302	-0.290	-0.261	-0.036	0.307	0.215	-3.294	0.171	0.000
Chibby protein (PKD2 interactor, Golgi and endoplasmic reticulum associated 1) (PIGEA-14) (Cytosolic leucine-rich protein) (HRIH	CN952220	0.000	-0.464	0.657	-0.244	0.566	0.423	1.834	1.724	-1.534	0.724	0.005
NA	CN854450	0.000	-0.342	-0.322	-1.095	-0.315	-0.037	-0.448	-0.117	-0.788	-0.510	0.002
NA	EX568269	0.000	0.693	0.176	0.603	0.689	0.865	0.392	0.994	-1.795	0.564	0.004
NA	FE659471	0.000	0.270	0.503	0.134	0.490	0.568	0.787	0.638	-1.480	0.159	0.002
NA	FE840910	0.000	-0.106	-0.381	-0.447	-0.428	-0.203	-0.262	-0.400	-0.772	-0.143	0.005
NA	DV772553	0.000	0.313	-0.111	0.710	0.053	0.461	-0.580	0.708	-0.358	0.516	0.004
NA	EX471088	0.000	0.988	0.763	1.163	2.080	0.853	1.448	1.404	-1.880	1.314	0.002
NA	EY116761	0.000	0.177	0.636	0.656	0.202	0.558	0.997	0.267	-2.582	0.546	0.003
NA	EX486606	0.000	-2.821	-4.238	-2.140	-2.909	-2.343	-1.933	-2.979	-5.676	-2.365	0.002
NA	DV771280	0.000	0.815	1.225	1.108	1.643	0.805	2.517	1.457	-1.443	0.590	0.002
NA	EX827425	0.000	-0.625	-0.188	-0.500	-0.424	-0.322	-0.443	-0.454	-0.741	0.063	0.002
reticulocalbin [Tribolium castaneum]	EY290811	0.000	0.716	-0.095	-0.152	0.632	0.638	0.458	0.574	-1.088	-0.197	0.002
hypothetical protein AaeL_AAEL010280 [Aedes aegypti]	FE535372	0.000	0.161	0.943	-2.007	0.270	0.626	-0.894	-0.108	-2.553	-0.861	0.004
glucosamine-6-phosphate N-acetyltransferase [Bombyx mori]	EH116667	0.000	-0.517	0.432	-0.366	-0.136	-0.149	0.266	0.063	-5.442	1.410	0.000
cellular repressor of E1A-stimulated genes [Apis mellifera]	FE043722	0.000	0.237	0.292	-0.275	0.235	0.157	-0.361	0.229	-0.688	-0.193	0.003
Clast3 protein [Nasonia vitripennis]	CN853293	0.000	-0.176	0.170	-0.025	0.028	-0.070	-0.374	-0.119	-0.570	-0.097	0.001
minor ampullate silk protein MiSp1 [Nephila clavipes]	DV773512	0.000	-0.242	0.381	-0.072	-0.125	-0.065	-0.216	-0.112	-0.619	-0.025	0.002
Phosphoenolpyruvate carboxykinase [Nephrops norvegicus]	DV771445	0.000	0.797	0.961	0.157	-0.074	-0.086	-0.131	-0.138	-1.899	-0.217	0.000
NA	EH116131	0.000	0.128	0.207	-0.176	-0.464	-0.232	-0.428	-0.165	-0.930	-0.355	0.002

Cluster 7 (18 genes)

Gene Name or best BLASTx hit	Accession #	24h Control	24h Infected	48h Infected	1 Week Infected	3-4 Week Infected	5-7 Week Infected	8 Week Infected	10 Week Infected	8 Week Control	10 Week Control	p-value
pol-like protein [Biomphalaria glabrata]	FE659743	0.000	-0.084	-1.426	-0.482	-0.221	-0.342	-0.567	-0.304	-0.948	-0.190	0.003
ALFHa-04	EX568422	0.000	-0.360	-0.476	-0.191	0.213	0.710	1.255	1.236	-0.321	-0.515	0.005
Toll-like receptor (AGAP012385-PA) [Anopheles gambiae str. PEST]	FE535091	0.000	0.614	-0.170	0.686	0.656	0.456	1.136	1.033	-0.705	0.660	0.004
NA	FE043642	0.000	-0.388	-0.573	-0.826	-0.178	0.008	0.567	0.023	-0.883	-0.549	0.003
NA	EW997809	0.000	0.402	-0.484	-0.074	0.192	0.356	0.419	0.262	-0.227	0.732	0.005
NA	FE659547	0.000	0.876	-2.177	0.697	0.568	0.816	0.490	0.685	-0.604	0.932	0.004
upstream stimulatory factor 1 [Mus musculus]	EH116864	0.000	-0.298	-1.168	-0.145	-0.092	-0.460	-0.521	-0.033	-0.693	-0.027	0.002
ALFHa-6	DV774220	0.000	0.453	-0.212	0.608	1.011	1.367	1.980	1.922	-0.453	0.544	0.000
alanine-glyoxylate transaminase 1 [Glossina morsitans morsitans]	EG949095	0.000	0.509	-0.595	0.610	0.605	0.719	0.863	0.532	-0.369	0.707	0.001
acute phase serum amyloid A (SAA) [Oncorhynchus mykiss]	CN949986	0.000	-0.011	-0.447	-0.416	0.586	0.810	0.847	1.457	-0.412	-0.453	0.000
NA	FD467831	0.000	0.338	-0.773	0.029	0.239	0.298	0.101	0.639	-0.780	0.368	0.000
NA	EX487335	0.000	-0.722	-0.781	-0.584	-0.563	-0.307	-0.312	-0.320	-0.687	-0.234	0.001
predicted protein [Nematostella vectensis]	CN950219	0.000	0.080	-0.472	0.110	0.145	0.124	-0.406	0.175	-0.192	0.057	0.004
Zinc phosphodiesterase ELAC protein 2 (Ribonuclease Z 2) (RNase Z 2) (tRNase Z 2) (tR 3 endonuclease 2) (ElaC homolog protein 2)	FD699226	0.000	-0.171	-1.662	-0.190	0.216	-0.234	-0.316	-0.333	-0.843	-0.052	0.002
Radical S-adenosyl methionine domain containing 1 [Homo sapiens]	FD467478	0.000	-0.746	-0.481	-0.044	0.120	-0.036	0.001	-0.072	-0.401	0.086	0.000
CG8486-PA, isoform A [Apis mellifera]	CN951386	0.000	-0.573	-2.011	-0.941	-0.275	-1.229	-1.097	-1.250	-1.153	-1.001	0.005
NA	CN853019	0.000	-0.551	-0.726	-0.089	0.004	-0.335	-0.503	-0.064	-0.864	-0.197	0.001
UDP-glucose 6-dehydrogenase [Aedes aegypti]	FE535832	0.000	-0.032	-1.019	-1.201	0.142	0.184	-0.509	0.181	-1.611	-0.510	0.001

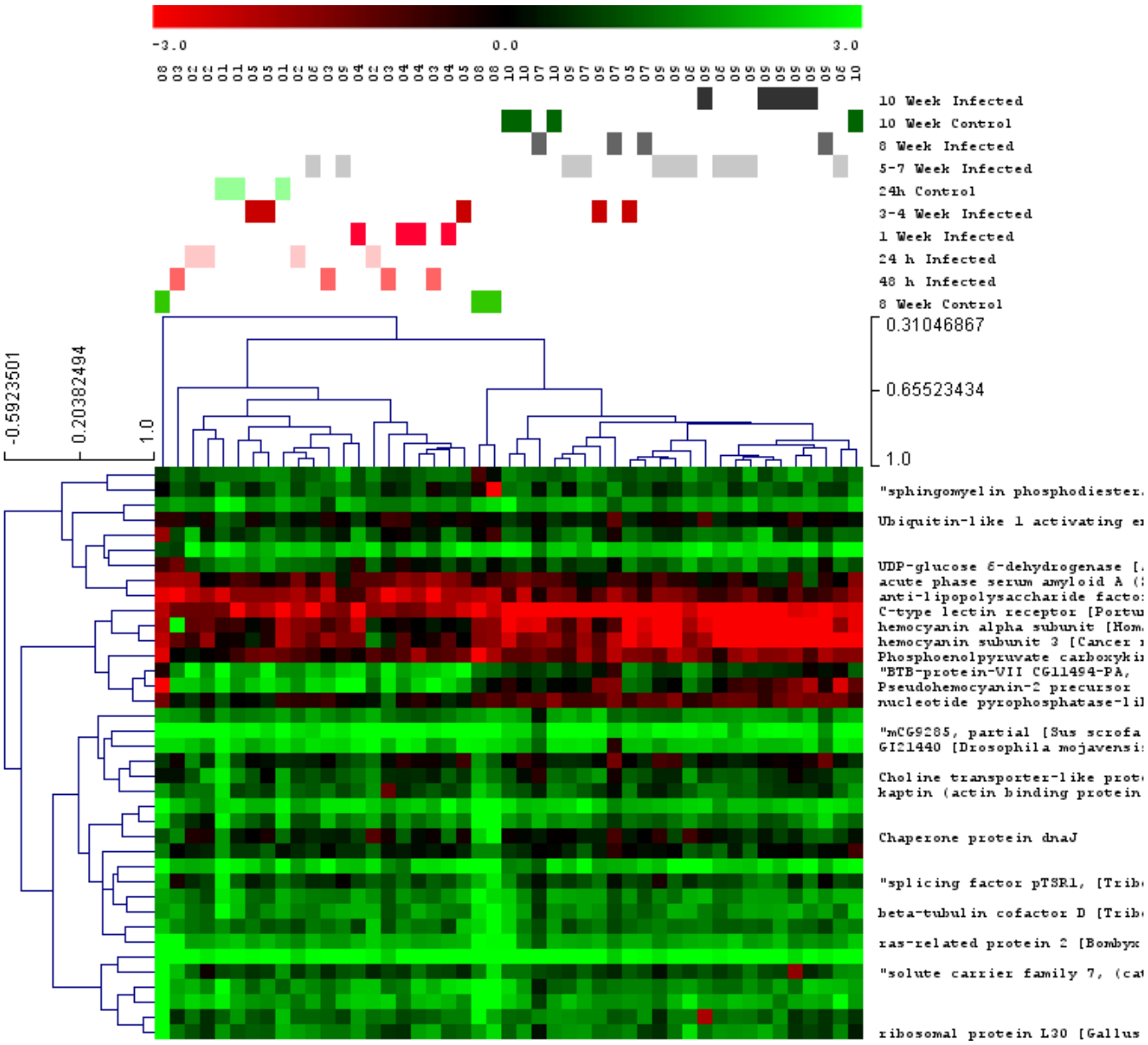

Figure B.1 Hierarchical Clustering of the 38 differentially expressed genes at Bonferroni corrected $\alpha = 0.005$, during *Anophryoides haemophila* infection. Gene expression heat maps of the ratio of gene expression where a gradient of red to green represents a three-fold or greater decrease in gene expression to a three-fold or greater increase in gene expression. Columns represent the average gene expression of a given treatment at each gene displayed horizontally.

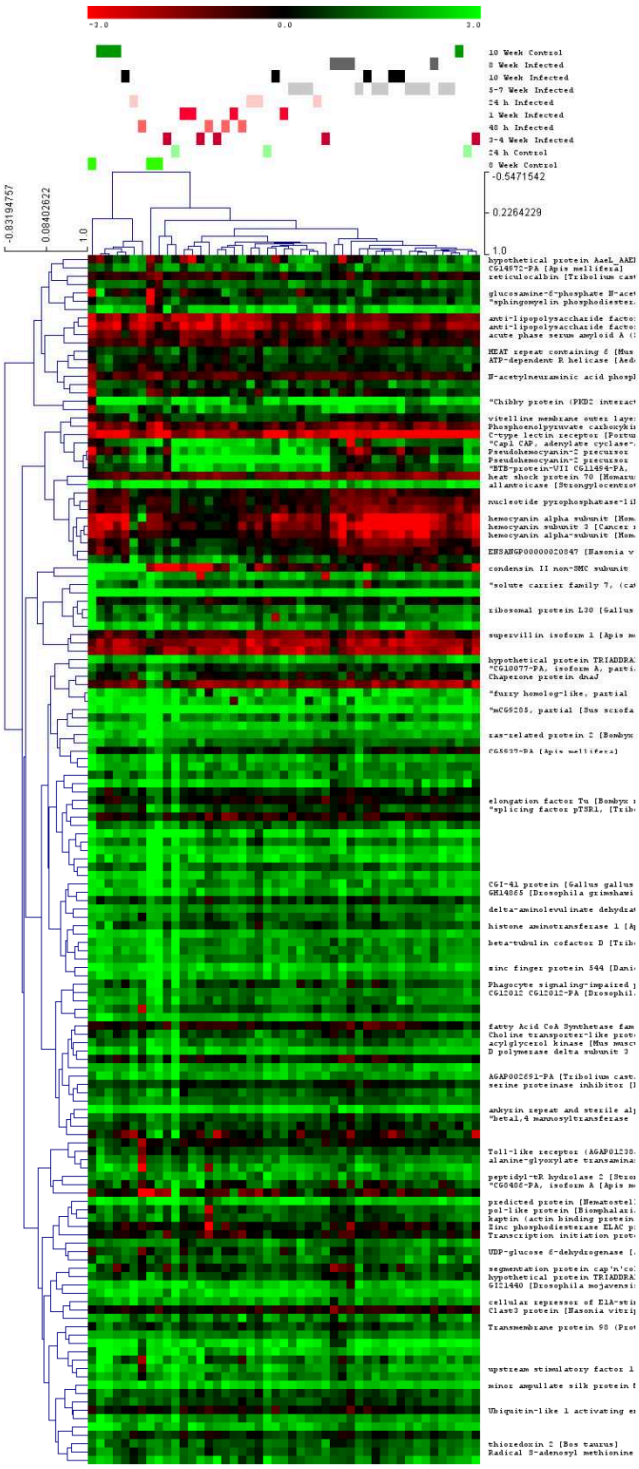

Figure B.2 Hierarchical Clustering of the 145 differentially expressed genes at $\alpha = 0.005$, during *Anophryoides haemophila* infection. Gene expression heat maps of the ratio of gene expression where a gradient of red to green represents a three-fold or greater decrease in gene expression to a three-fold or greater increase in gene expression. Columns represent the average gene expression of a given treatment at each gene displayed horizontally.

Appendix C

Table C.1 *Homarus americanus* genes differentially expressed as measured by microarray and ANOVA at a significance level of a = 0.005. Expression is listed as the log2 ratio of sample/reference relative to the 6 h control. Clusters have been generated by K-means clustering.

Cluster 1	28 genes									
Shares most translated sequence similarity with:	Accession #	6 h Control	168 h Control	6 h Infected	12 h Infected	24 h Infected	48 h Infected	96 h Infected	168 h Infected	p-value
adenylate cyclase [Tribolium castaneum]	CN853934	0.00	-0.47	-0.5	-2.18	-1.75	-0.94	-1.1	-0.75	0
NA	CN950777	0.00	0.46	0.3	-0.73	-1.24	-0.52	-0.03	0.02	0
NA	CN951004	0.00	-1.1	0.13	-0.89	-1.69	-0.22	-0.5	-0.13	0
NA	FE660144	0.00	-0.2	-0.21	-1.62	-1.69	-1.43	-0.64	-0.19	0.01
Kazal-like serine protease inhibitor EPI9 [Phytophthora infestans]	FE535180	0.00	0.12	0.62	-0.33	-0.71	0.2	0.9	0.6	0
NA	FE841501	0.00	-1.52	-0.25	-1.41	-2.11	-1.02	-0.93	-0.69	0
NA	DV774303	0.00	-0.16	0.24	-0.74	-1.22	-0.34	0.33	0.26	0
pyruvate dehydrogenase [Nasonia vitripennis]	EG948623	0.00	-0.39	0.02	-0.64	-0.67	0.46	0.29	0.53	0
NA	EG948951	0.00	-1.33	0.04	-1.29	-1.7	-0.99	-1.16	-0.75	0
NA	EG949190	0.00	-1.45	-0.52	-1.42	-1.23	-0.89	-0.81	-0.46	0.01
NA	FD483589	0.00	-0.34	-0.22	-0.96	-1.57	-0.67	-0.57	-0.42	0
NA	EH117249	0.00	-0.57	0.27	-1.53	-2.42	-0.46	-0.05	0.1	0
ATP synthase F0 subunit 6 [Amblyraja radiata]	FE535158	0.00	-0.78	0.26	-1.17	-1.77	-0.98	-0.3	0.61	0
NA	EX827407	0.00	0.59	0.48	-0.6	-1.13	-0.23	0.48	0.96	0
NA	EY290828	0.00	-1.48	0.27	-1.03	-1.84	-1.28	-1.05	-0.71	0
NA	EY291014	0.00	-0.5	0.2	-1.01	-1.09	0.05	-0.42	-0.16	0
NA	FC071437	0.00	0.11	0.34	-0.5	-0.95	0.11	0.97	0.05	0
"Gelsolin, cytoplasmic (Actin-depolymerizing factor) (ADF)"	FC071845	0.00	-1.05	0.41	-0.49	-1.41	-0.99	-0.14	-0.19	0
tetraspanin [Tribolium castaneum]	FD425482	0.00	-0.82	-0.11	-1.67	-1.68	-1.16	-0.18	0.06	0
Oxysterol-binding protein-related protein 6 (OSBP-related protein 6) (ORP-6)	FE535126	0.00	-0.31	0.03	-1.01	-1.47	0.15	0.05	0.59	0
NA	FD584934	0.00	-0.17	0.79	-0.73	-1.31	-0	0.02	0.42	0
NA	FD699702	0.00	-0.42	0.55	-0.8	-0.95	-0.45	0.05	-0.11	0
NA	FE043548	0.00	-0.88	0.38	-0.57	-1.24	-0.16	-0.32	0.9	0
"apoptosis-inducing factor like, [Monodelphis domestica]"	FE043558	0.00	-0.44	-0.53	-1.61	-1.24	-0.21	0.11	-0.05	0
NA	FE043699	0.00	-0.84	-0.07	-1.12	-1.03	-0.58	-0.09	-0.24	0
NA	FE043725	0.00	-0.61	0.37	-1.01	-1.43	-0.64	0.01	0.11	0.01
"Cell wall mannoprotein with similarity to Tir1p, Tir2p, Tir3p, and Tir4p	FE659742	0.00	-0.43	0.17	-0.87	-1.61	-0.52	0.27	0.35	0
NA	FE840989	0.00	0.66	0.13	-0.96	-0.78	0.13	1.25	0.89	0

Cluster 2	44 genes									
Shares most translated sequence similarity with:	Accession #	6 h Control	168 h Control	6 h Infected	12 h Infected	24 h Infected	48 h Infected	96 h Infected	168 h Infected	p-value
ribosomal protein S3 [Homo sapiens]	DV775052	0.00	1.97	0.23	1.02	0.8	0.32	0	-0.36	0
bicaudal CG3644-PA isoform 1 [Tribolium castaneum]	EH116482	0.00	1.5	0.3	1.16	0.28	-0.38	-0.15	-0.45	0
splicing factor 45 [Tribolium castaneum]	FE841480	0.00	1.47	-0.49	0.65	0.1	0.1	-0.31	-0.34	0
NA	FD467297	0.00	1.18	0.23	1.14	0.54	-0.23	-0.41	0.37	0.01
ribosomal protein S15 [Barentsia elongata]	CN854356	0.00	2.23	0.67	0.79	0.5	-0.03	-0.21	0.15	0.01
Digestive cysteine proteinase 1 precursor	CN951236	0.00	1.46	-0.32	0.13	0.47	-0.48	-0.59	-0.98	0
60S ribosomal protein L7A [Ixodes pacificus]	EH035698	0.00	2.02	0.05	0.7	0.69	-0.24	0.13	0.08	0
60S ribosomal protein L38 [Brugia malayi]	FE841504	0.00	2.88	-0.12	1.64	0.69	0.22	0.58	-0.27	0
ribosomal protein L13 [Danio rerio]	FE535088	0.00	1.77	-0.14	0.65	0.81	0.01	0.28	-0.24	0
"exonuclease 3-5 domain-like 2, [Monodelphis domestica]"	CN853396	0.00	1.74	0.82	0.85	-0.04	0.05	0.02	-0.23	0
oxidase\peroxidase [Tribolium castaneum]	CN853665	0.00	1.32	0.13	-0.14	0.44	0.1	-0.52	-0.55	0
ribosomal protein L36e isoform 2 [Acyrthosiphon pisum]	CN853904	0.00	2.21	0.05	0.98	0.28	-0.83	-0.25	-0.48	0
Ribosomal protein L4 CG5502-PA isoform 1 [Apis mellifera]	CN950680	0.00	2.15	0.26	1.11	0.62	0.44	-0.23	-0.24	0
eukaryotic translation initiation factor 3 subunit 5 epsilon-like [Ixodes scapularis]	EH035483	0.00	1.42	0.16	1.07	0.37	-0.28	-0.6	-0.33	0
Ribosomal protein L21 [Mus musculus]	CN854379	0.00	1.88	0.44	1.02	0.45	-0.58	-0.14	-0.2	0.01
NA	CN950029	0.00	2.11	0.17	0.27	0.1	-0.51	-0.45	-0.39	0
ribosomal protein L35 [Xenopsylla cheopis]	CN950217	0.00	2.32	0.66	1.08	0.57	0.28	0.37	0.13	0.01
ribosomal protein S19e [Eucinetus sp. APV-2005]	CN950722	0.00	2.18	-0.02	1.17	0.37	0.36	0.18	-0.2	0
ribosomal protein L30 [Argopecten irradians]	CN951693	0.00	1.76	-0.37	0.56	0.14	-0.18	0.01	-0.46	0
"60S acidic ribosomal protein P2, [Aspergillus clavatus NRRL 1]"	FE659818	0.00	2.26	1	1.43	0.65	-0.04	0.45	0.34	0
NA	DV771674	0.00	2.1	0.03	1.13	0.42	-0.39	-0.63	0.03	0
"squid CG16901-PC, isoform C isoform 1 [Apis mellifera]"	DV773131	0.00	1.62	-0.55	0.56	0.46	-0.46	0.17	-0.16	0
NA	DV773730	0.00	1.76	0.27	1.22	0.34	-0.6	-0.24	-0.28	0
ribosomal protein S18 [Cherax destructor]	FE841449	0.00	2.38	-0.32	0.73	0.77	0.18	0.29	-0.4	0
Ribosomal protein L37a CG9091-PA [Drosophila melanogaster]	FD699124	0.00	2.74	-0.02	1.17	0.9	0.24	0.4	-0.45	0
ribosomal protein S25 [Ixodes scapularis]	FC556272	0.00	2.47	-0.15	0.7	0.23	-0.66	0.11	-0.39	0
zinc finger protein 452 [Acyrthosiphon pisum]	EH034796	0.00	4.24	0.24	0.71	0.85	-0.16	-0.27	-0.62	0
ribosomal protein S24 [Marsupenaeus japonicus]	EX827155	0.00	2.57	-0	1.09	0.61	-0.09	0.16	0.03	0
cathepsin C [Marsupenaeus japonicus]	EH116404	0.00	1.68	0.22	1.05	1.24	0.28	-0.8	-1	0
peritrophic membrane chitin binding protein 2 [Trichoplusia ni]	EH116415	0.00	1.5	-0.72	-0.13	-0.11	-0.59	-0.54	-0.87	0
NA	EH401315	0.00	1.38	-0.13	0.86	0.39	-0.36	-0.3	-0.16	0
60S ribosomal protein L37a	FE535452	0.00	2.67	-0.66	1.17	0.41	-0.24	0.06	-0.36	0
trypsin [Litopenaeus vannamei]	EV781656	0.00	1.45	-0.55	-0.3	-0.45	-0.7	-1.22	-1.59	0

ribosomal protein L28-like protein [Maconellicoccus hirsutus]	FE841076	0.00	2.34	-0.22	0.65	0.29	-0.38	0.39	-0.62	0
"Chitobiase, di-N-acetyl- [Danio rerio]"	FD699488	0.00	1.88	-0.12	0.37	-0.33	-0.56	-0.68	-0.6	0
NA	EY290738	0.00	1.66	0.61	0.48	0.81	0.57	0.32	-0.08	0
"acyl-Coenzyme A dehydrogenase family, member 9 [Gallus gallus]"	FC556690	0.00	1.35	0.78	0.14	0.29	-0.25	-0.41	-0.98	0
NA	FE841209	0.00	1.88	-0	0.68	1.32	0.61	0.79	0.25	0.01
ubiquitin\ribosomal protein S30e fusion protein [Hister sp. APV-2005]	FF277633	0.00	2.8	0.38	1.51	0.74	-0.25	-0.06	-0	0.01
NA	CN852900	0.00	1.35	1.39	0.66	-0.11	-0.25	0.32	-0.67	0
Rpl23a protein [Pan troglodytes]	FF278016	0.00	2.31	0.3	0.9	0.55	0.04	-0.2	-0.62	0
endonuclease and reverse transcriptase-like protein [Bombyx mori]	FE841037	0.00	3.01	0.11	0.41	1.22	-0.57	-0.28	-0.37	0
ribosomal protein L23 [Haemaphysalis qinghaiensis]	FF278016	0.00	2.06	-0.27	0.48	0.39	-0.11	-0.01	-0.48	0
NA	FD584511	0.00	2.5	0.78	2.39	1.94	1.2	1.69	1.25	0

Cluster 3	36 genes									
Shares most translated sequence similarity with:	Accession #	6 h Control	168 h Control	6 h Infected	12 h Infected	24 h Infected	48 h Infected	96 h Infected	168 h Infected	p-value
splicing factor 3B subunit 4 [Culex quinquefasciatus]	FE044289	0.00	1.28	0.76	-0.39	-0.64	-0.52	0.55	0.05	0
ATP synthase F0 subunit 6 [Cherax destructor]	FF277325	0.00	1.24	-0.31	-0.56	-0.66	-0.99	0.06	-0.15	0
NA	EY116930	0.00	0.76	-1.07	-0.6	-1.23	-1.43	-0.89	-1.86	0
NA	CN949854	0.00	1.15	-0.14	-1.13	-1.07	-0.99	0.08	-1.01	0
NA	CN949886	0.00	1.85	-0.59	-0.8	-0.6	-1.04	-0.71	-0.8	0
cytochrome c oxidase subunit III [Pseudocarcinus gigas]	FD467412	0.00	1.42	-0.53	-0.4	-0.56	-0.74	0.26	-0.35	0
juvenile hormone esterase [Acyrthosiphon pisum]	CN950668	0.00	1.73	-0.56	0.04	-1.39	-1.38	-1.26	-1.46	0
NA	CN951882	0.00	1.57	-0.36	-0.57	-0.55	-0.14	-0.15	-1.27	0
ribosomal protein L11 [Ixodes scapularis]	CN952190	0.00	0.94	-0.37	-0.4	-0.26	-0.43	-0.3	-0.67	0
DH dehydrogenase subunit 3 [Geothelphusa dehaani]	DV774077	0.00	1.47	-0.31	0.02	-0.36	-0.57	0.17	-0.83	0
NA	DV774386	0.00	1.16	-0.59	-0.06	-0.8	-0.92	-0.46	-0.96	0
60S ribosomal protein L15	EX486674	0.00	1.77	-0.31	-0.56	-0.47	-0.66	-0.16	-0.65	0
NA	DV771836	0.00	1.04	-0.81	-0.49	-0.62	-0.77	-0.19	-1.44	0
NA	DV773312	0.00	0.7	-0.71	-1	-0.66	-0.76	-0.36	-0.73	0
40S ribosomal protein SA [Diaphorina citri]	GH709959	0.00	1.11	-0.52	-0.4	-0.64	-0.36	-0.39	-0.72	0
NA	EG949447	0.00	0.42	-0.15	-0.94	-1.45	-0.79	-0.29	-0.4	0
NA	EG949642	0.00	1.54	-0.59	-0.44	-0.56	-0.16	0.74	-0.27	0
ribosomal protein S5 isoform 1 [Apis mellifera]	FD467527	0.00	0.73	-0.5	-0.78	-0.67	-0.62	-0.2	-0.78	0
NA	EG949713	0.00	0.48	0.15	-0.28	-0.52	-0.26	0.21	-0.21	0
NA	EH034751	0.00	0.87	0.28	-0.68	-0.65	-0.45	0.36	-0.21	0
NA	EH034876	0.00	0.96	-0.66	-0.65	-0.51	-0.45	0.13	-1.1	0
NA	EH035460	0.00	1.06	-1.16	-0.78	-0.62	-1.31	-1.15	-1.84	0
NA	EH116521	0.00	0.93	0.15	-0.28	-0.6	0.26	0.29	-0.16	0.01
beta actin [Pan troglodytes]	EH401642	0.00	0.67	-0.55	-1.06	-2.11	-1.81	-0.73	-1.37	0
NA	EW997761	0.00	0.51	-0.4	-0.96	-1.41	-0.72	-0.39	-0.22	0
Ecdysone-inducible protein E75 (Nuclear receptor subfamily 1 group D member 3)	EX471223	0.00	0.98	-0.47	-1.14	-0.77	-0.75	0.12	-0.46	0
NA	EX487073	0.00	1.15	0.22	0.04	-0.37	-0.22	0.13	-0.58	0
ribosomal protein rpl17 [Arenicola marina]	EY117358	0.00	1.48	-0.51	-0.3	-0.66	-0.41	-0.28	-0.63	0
NA	FD483488	0.00	0.4	0.11	-0.9	-0.6	-0.01	-0.15	-0.36	0
NA	FD699340	0.00	0.44	-0.21	-0.7	-0.79	-0.4	-0.03	-0.74	0
NA	FD699814	0.00	0.63	0.08	-0.69	-0.82	-0.97	-0.09	-0.43	0.01
"Darkener of apricot CG33553-PB, isoform B [Drosophila melanogaster]"	FE043587	0.00	1.73	-0.21	-0.92	-0.77	-0.91	-0.12	-0.93	0
NA	FE044136	0.00	0.33	-1.09	-1.31	-0.9	-0.84	-0.66	-1.28	0
NA	FE659950	0.00	0.66	-0.43	-1	-1.07	-0.99	-0.16	-0.7	0
ATP synthase subunit 9 mitochondrial precursor [Litopenaeus vannamei]	FE535328	0.00	1.86	-0.44	-0.15	-0.55	-0.35	0.8	0.35	0
NA	EG949384	0.00	0.38	-1.06	-0.81	-2.16	-1.48	-0.76	-1.25	0

Cluster 4	17 genes									
Shares most translated sequence similarity with:	Accession #	6 h Control	168 h Control	6 h Infected	12 h Infected	24 h Infected	48 h Infected	96 h Infected	168 h Infected	p-value
NA	CN852804	0.00	2.3	1.12	0.79	0.22	0.53	0.34	0.38	0
fibrillarin [Mus musculus]	CN853706	0.00	2.36	-0.03	0.55	0.06	-0.28	-0.08	-0.15	0
"Transport and Golgi organization 5 CG32675-PB, isoform B [Drosophila melanogaster]"	FE535594	0.00	1.44	0.36	0.37	-0.58	0.37	0.56	0.36	0
60S ribosomal protein L7a [Lepeophtheirus salmonis]	CN950445	0.00	2.24	-0.2	0.61	0.13	0.41	0.2	-0.54	0.01
ribosomal protein L10 [Callinectes sapidus]	FE043814	0.00	2.47	-0.2	0.43	0.27	0.08	0.17	-0.04	0
ribosomal protein S26 [Branchiostoma belcheri]	FE659958	0.00	1.98	-0.3	0.27	-0.41	-0.49	0.29	-0.42	0.01
NA	DV772642	0.00	3.58	0.14	0.86	-0.13	-0.11	-0.31	-0	0
NA	DV774072	0.00	2.59	-0.63	0.81	-0.08	-0.26	0.24	-0.33	0
ribosomal protein L35a [Homo sapiens]	EX487234	0.00	2.34	-0.12	0.5	-0.09	-0.27	0.42	-0.69	0
"abrupt CG4807-PA, isoform A [Drosophila melanogaster]"	EH035734	0.00	5.45	0.23	0.56	-0.69	-0.66	0.3	-0.77	0
acetyl-CoA synthetase 2 [Strongylocentrotus purpuratus]	FD585373	0.00	4.01	0.34	-0.12	0.3	-0.47	-0.57	0.26	0
ribosomal protein S4e [Graphocephala atropunctata]	FF278021	0.00	2.71	0.22	1.16	0.41	0.08	0.38	0.22	0
cytochrome P450 [Orconectes limosus]	EX487185	0.00	6.28	0.19	0.11	1.01	-0.22	-0.24	-0.05	0
60S ribosomal protein L18a [Triatoma infestans]	FD699452	0.00	2.67	0.46	0.96	0.36	0.61	0.99	0.46	0
NA	EX568609	0.00	2.33	0.03	0.28	0.59	0.49	0.7	0.1	0.01
NA	FE044026	0.00	3.68	-0.1	0.86	0.11	0.1	0.71	-0.12	0
B-box zinc finger family protein [Tetrahymena thermophila SB210]	FE659655	0.00	0.99	-0.59	-0.24	-0.28	-0.12	-0.45	-0.08	0

Cluster 5	11 genes									
Shares most translated sequence similarity with:	Accession #	6 h Control	168 h Control	6 h Infected	12 h Infected	24 h Infected	48 h Infected	96 h Infected	168 h Infected	p-value
NA	CN853585	0.00	0.79	0.16	1.55	-0.33	0.3	0.37	0.8	0
NA	CN854083	0.00	-0	-0	-0	0	0	0	0	0
AGAP007607-PA [Anopheles gambiae str. PEST]	EY290669	0.00	-0.07	1.8	1.35	-0.02	-0.36	0.05	0.86	0
NA	CN950869	0.00	-0.34	1.19	1.47	-0.07	-0.29	0.7	0.27	0.01
NA	EV781938	0.00	-0.07	0.35	1.36	-0.09	0.92	1.42	1.31	0.01
NA	DV773940	0.00	-1.23	1.16	1.05	0.71	0.5	0.42	0.29	0

NA	EH035399	0.00	1.31	2.72	3.03	2.13	2.83	2.44	1.72	0
NA	EH401735	0.00	0.04	0.84	1.39	0.72	0.35	0.22	-0.11	0
NA	FC556582	0.00	-1	0.14	1.7	0.16	-0.24	-0.75	-0.09	0
vacuolar proton atpases isoform 2 [Nasonia vitripennis]	FD483331	0.00	-0.44	1.64	1.48	0.43	1.23	0.6	0.02	0
ring finger protein 181 [Xenopus (Silurana) tropicalis]	FE841448	0.00	1.9	1.39	2.59	1.39	1.71	1.46	1.94	0